

# STAT1 and IRF-mediated Signal Integration of IFN $\gamma$ and TLR4 in Vascular Inflammation

doctoral thesis

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# Integracja szlaków sygnalizacyjnych interferonu gamma i receptora TLR4 w procesie zapalnym naczyń krwionośnych, warunkowana czynnikami transkrypcyjnymi STAT1 i IRF

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# Chapter 1

## Introduction

Cardiovascular diseases (CVDs), a group of disorders that affect the heart and blood vessels, are globally the leading cause of death. According to the World Health Organization, the number of people that will die annually from CVDs will increase from 17.3 million (2008) to 23.3 million in 2030 [1, 2]. Recently, several studies have identified an immune component as an important factor linking a distinct set of lifestyle elements that is involved in CVDs progression and that plays a significant role in the pathophysiology of CVDs [3].

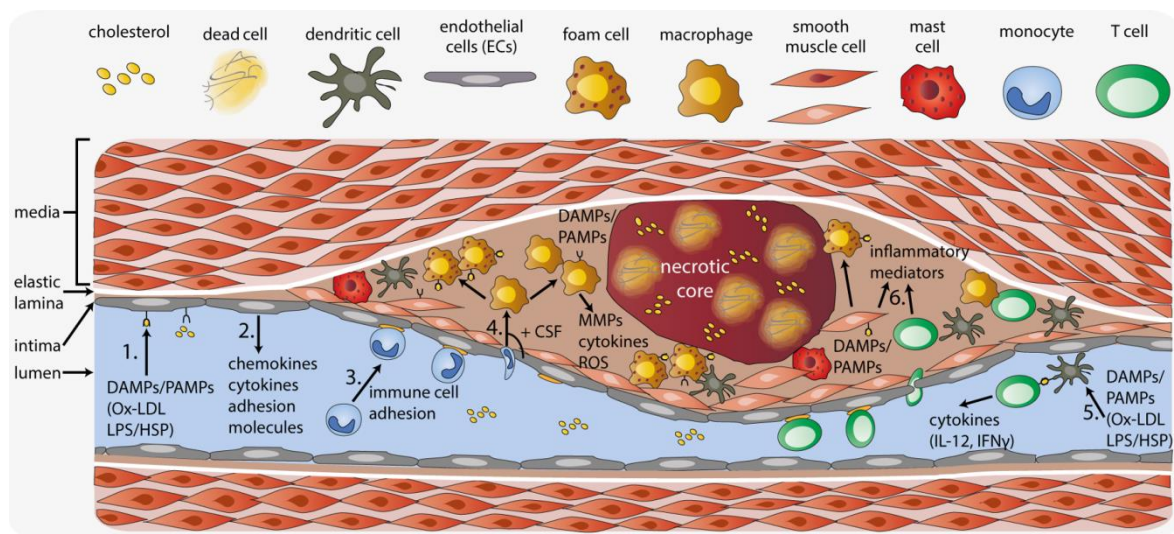
During the evolutionary process animals developed a highly complex system that maintains internal homeostasis. The immune system protects organisms against exogenous pathogens and enables repair of tissue damage caused by infection or trauma. This system can be divided into two strictly connected categories: innate and adaptive. The innate immune system, through a network of distinct pathogen recognition receptors (PRRs), recognizes pathogen-associated molecular patterns (PAMPs) and creates the first line of defense [4]. Activation of the immune system leads through a distinct set of effector cells (e.g. macrophages, NK cells, dendritic cells) to an acute response which is nonspecific and limited to a number of irritants [5]. On the other hand, the adaptive immune system which consists of several subsets of T cells and B cells is far more diverse and refers to an antigen-specific immune response. Cytokines play a crucial role in regulating the immune response. These small proteins, expressed by different types of cells, can either stimulate proinflammatory responses (e.g.  $IL1\beta$ ,  $IFN\gamma$ ,  $TNF\alpha$ ) or suppress them (e.g.  $IL10$ ,  $TGF\beta$ ).

In spite of the immune system's complexity in certain conditions, the inflammatory response may damage host tissue and participate in pathophysiology of the disease [6]. In some cases, e.g. sepsis, the acute and systemic immune response may cause multiple organ dysfunction [7]. In other diseases, loss of immunological tolerance to self-antigens, described as autoimmunity, plays an important role in progression of illness. This is a major health issue, as autoimmunity has been identified as a contributing factor of 80 different disorders that collectively impact 4-7% of the population in the United States [8]. Moreover, in many disorders that are related to CVDs, prolonged exposure to potentially toxic agents creates



damage-associated molecular patterns (DAMPs) that trigger chronic inflammation which affects progression of a disease [9].

Chronic inflammation participates in the development of atherosclerosis, which is a leading cause of coronary artery disease (CAD) [10]. Pathomechanism of this disorder consists of endothelial dysfunction with leukocyte recruitment, de-differentiation of vascular smooth muscle cells and asymmetrical focal thickening of the vessel wall [11]. Over time, initial lesions may transform into fully developed atheroma characterized by the presence of different cell types and a lipid-rich core surrounded by a fibrotic cup (Fig. 1-1).



**Fig. 1-1. Involvement of inflammation in the plaque development.** 1, In stress conditions, DAMPs and PAMPs are created. These ligands provoke innate immune responses in the arterial wall and increase the permeability of the blood vessels for lipoproteins. 2, Activation of the ECs leads to the expression of chemokines, cytokines which enhance adhesion and extravasation of the leukocytes (3). 4, Activated endothelial cells express macrophage colony-stimulating factor (CSF) and cause differentiation of the attracted monocytes into macrophages. Intimal macrophages can not only take-up oxLDL which transform them into foam cells but also express cytokines, matrix metalloproteinases (MMPs) and reactive oxygen species (ROS). 5, Other professional antigen-presenting cells, such as dendritic cells (DCs), can uptake lipoproteins and other DAMPs in order to present them on the cell surface for recognition by the T cells. Activated T cells produce inflammatory cytokines e.g. IFN $\gamma$  or IL12. 6, Smooth muscle cells upon change their phenotype from contractile to synthetic and start to proliferate and produce inflammatory mediators.

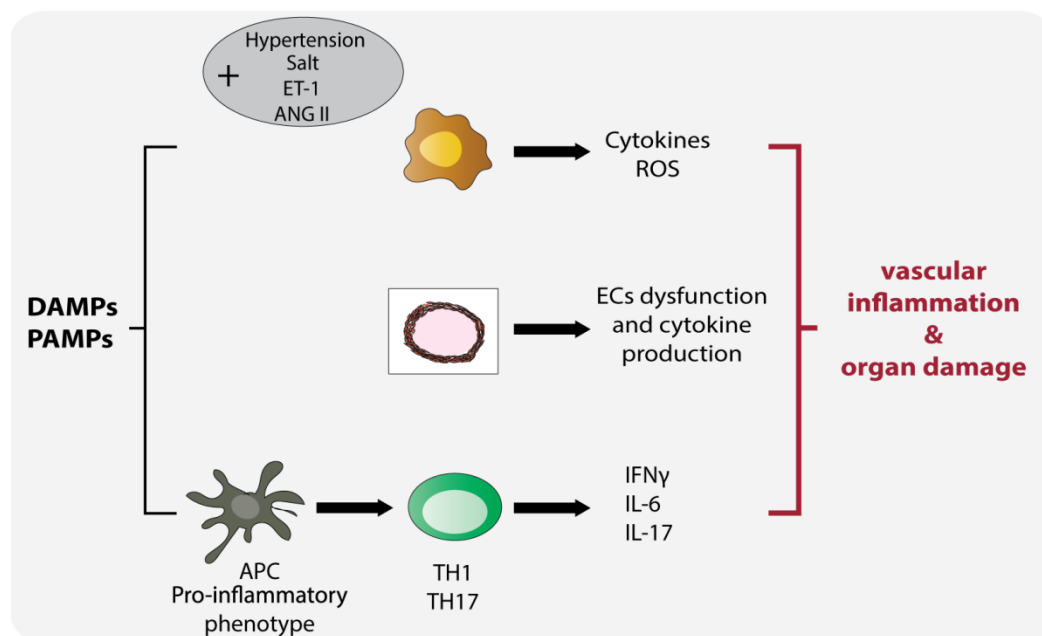
Atherosclerotic plaque can narrow the lumen of the vessel and cause ischemia of the surrounding tissue [12]. Furthermore, in certain conditions the plaque may rupture and release the content of the necrotic core which triggers blood clot formation in the vessel (thrombosis). A thrombus may cause stenosis of the vessel or can detach and become an embolus that can block the flow of blood distant from its point of origin [12].

In a healthy vessel the function of the endothelial cells (ECs) is to maintain vascular homeostasis by regulating blood flow and creating a semi-selective barrier between the lumen and surrounding tissue. At this stage the ECs exert anticoagulant, antiplatelet and fibrinolytic properties [13]. However, in stress conditions (e.g. dyslipidemia, hypertension or diabetes), DAMPs and PAMPs are created. These ligands provoke innate immune responses in the arterial wall and increase the permeability of the blood vessels for lipoproteins, such as for low-density lipoprotein (LDL) or its oxidized form called oxLDL. Indeed, it has been shown that activation of the ECs leads to the expression of adhesion molecules such as E-selectin and VCAM-1 which, together with CCL2 and CCL5 chemokines, begin to promote leukocyte adhesion and their recruitment to the vessel wall. Moreover, inhibition of these chemokines in hypercholesterolemic, atherosclerosis-susceptible apolipoprotein E-deficient mice leads to a 90% reduction in atherosclerosis [14-16]. Activated endothelial cells express the macrophage colony-stimulating factor and cause differentiation of the attracted monocytes into macrophages [17]. Intimal macrophages can take-up oxLDL that eventually transform them into foam cells. In this initial step an early lesion, called a fatty streak, consists of a subendothelial deposition of lipids, macrophage foam cells loaded with cholesterol and T cells [12]. Recent evidence indicates that not only the macrophages but also vascular smooth muscle cells (VSMCs) expressing scavenger receptors can uptake lipoproteins and thus significantly contribute to foam cell formation [18]. In addition, macrophages present at the lesion site play an important role in stimulation of the innate immune response. Endogenous danger ligands that accumulate during atherosclerotic plaque development activate the PRRs, including toll-like receptors (TLRs) or NOD-like receptors, thereby activating an inflammatory response [16]. Depending on the nature of the ligand, macrophages exhibit different phenotypes. Classically activated macrophages (called M1) are activated by ligands of TLR-like lipopolysaccharide (LPS) or cytokines, e.g. interferon (IFN)- $\gamma$ . These macrophages are enriched in progressing plaques and express high levels of pro-inflammatory cytokines, including IL1 $\beta$ , IL12, and TNF $\alpha$  as well as reactive oxygen and nitrogen species which increase oxidative stress in the vessel [16, 19]. In contrast to M1, alternatively activated macrophages (called M2) secrete anti-inflammatory cytokines (e.g. IL10) and seem to play a protective role in atherosclerosis [16, 20]. Not only macrophages but also other professional antigen-presenting cells, such as dendritic cells (DCs), can uptake lipoproteins and other DAMPs

in order to present them on the cell surface for recognition by the T cells [21, 22]. Several subsets of T cells have been identified in atheroma [22, 23]. Among them the best characterized has been the role of  $T_H1$  cells which, upon activation, express IFN $\gamma$ , enhance development of the atherosclerotic lesion and contribute to plaque rupture. IFN $\gamma$  activates not only monocyte macrophages and DCs but also ECs and VSMCs to secrete cytokines and chemokines as well as a large amount of reactive oxygen species (ROS) and matrix metalloproteinases [24]. These findings have been confirmed in animal models of atherosclerosis where  $T_H1$ -deficient mice had significantly reduced atherosclerotic lesion formation [25]. In contrast to  $T_H1$ ,  $T_{reg}$  cells suppress the immune response, resolve inflammation in the plaque and thus have an atheroprotective role [24]. The role of the other two  $CD4^+$  lymphocytes,  $T_H2$  and  $T_H17$ , remains unknown as there are some conflicting reports about their function in atherosclerosis [22]. Furthermore, recent studies pointed to the involvement of  $CD8^+$  T cells and B cells in atherosclerosis, however, here also their exact role is unknown [22, 24]. Taken together, numerous studies reveal the importance of inflammation in all stages of plaque development and allow to consider atherosclerosis as a chronic inflammatory disease.

Another recently uncovered example of organ damage involving inflammation is hypertension [26]. Hypertension is defined as a chronic medical state with elevated blood pressure (systolic  $\geq 140$  and/or diastolic  $\geq 90$ ). Although hypertension is a major risk factor for cardiovascular mortality and morbidity, etiology of this disease remains poorly understood [27, 28]. Besides atherosclerosis, recent studies point to the importance of innate and adaptive immunity in the progression of a pathological state caused by elevated blood pressure. Increased immune cell infiltration has been observed in different models of hypertension. Macrophage colony-stimulating factor-deficient mice remain normotensive and develop less vascular remodeling and oxidative stress despite angiotensin (Ang) II or DOCA salt treatment [29, 30]. Ang II and DOCA salt-induced hypertension was ameliorated in T and B cell deficient mice [31]. Moreover, treatment with immunosuppressive drugs such as mycophenolate mofetil attenuates hypertensive organ damage and reduces renal and vascular immune cell infiltration [8]. Other studies have shown that inhibition of the proinflammatory cytokines, e.g. TNF $\alpha$ , IL6 or IL17, protects animals in an Ang II-induced model of hypertension [8].

Despite the large amount of data implicating inflammation in hypertension, the exact mechanism of immune activation is poorly understood. It is believed that elevated blood pressure may trigger activation of PRR by DAMPs and promotes an innate immune response. Upon DAMPs stimulation, ECs and VSMCs change their function and produce cytokines and chemokines that enhance extravasation. Activated immune cells produce cytokines and reactive oxygen species that exacerbate tissue damage. Additionally, important regulators of blood pressure, such as endothelin (ET)-1 or Ang II, induce an adaptive immune response either through activation of the DCs or directly by acting on the T cells, such as T<sub>H</sub>1 cells. Activated T cells produce inflammatory cytokines, e.g. IFN $\gamma$ , thus enhancing low-grade inflammation which contributes to organ damage [31, 32] (Fig. 1-2).



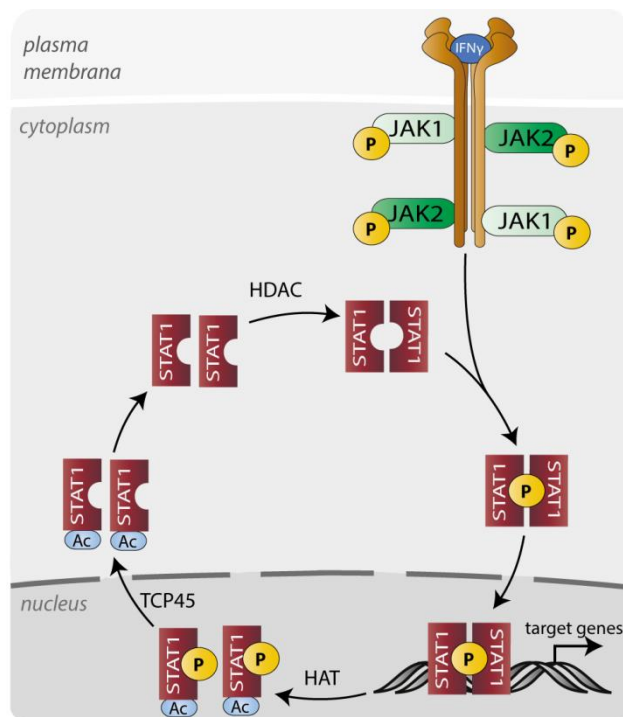
**Fig. 1-2. Role of inflammation in hypertension and hypertensive end organ damage.** Damage-associated molecular patterns (DAMPs) activate vasculature and components of the immune system leading to accelerated blood pressure increase and organ damage. Additionally regulators of blood pressure, such as ET-1 or Ang II, induce an adaptive immune response through activation of the dendritic cells (DCs) or directly by acting on the T cells [26].

Many of these cytokines, PAMPs and DAMPs (activators of TLRs) have shown to trigger the JAK/STAT pathway which is one of the pivotal pathways that operates at the frontier of innate and adaptive immunity and orchestrates the immune response [33]. Activation of this pathway with IFN $\gamma$  triggers a signal transduction cascade that modulates inflammation and as such has a prominent role in cardiovascular diseases. Although not completely

understood, the significance of the JAK/STAT pathway in chronic inflammatory processes has recently been recognized in immune cells [34]. Far less is known about the contribution of the JAK/STAT pathway in immunomodulatory functions of other non-immune cells [35, 36]. Considering the fact that non-immune cells such as ECs and vascular smooth muscle cells (VSMCs) are actively involved in the progression of inflammation, a better understanding of the processes behind a non-immune cells activation will have a substantial clinical benefit.

### IFN $\gamma$ Signaling Pathway

Interferons were discovered by Isaacs and Lindermann in the late 1950s and were initially considered as compounds that interfere with viral infection [37]. Later observations provided evidence for a more complex function of interferons, including anti-microbial responses, regulation of apoptosis, proliferation and regulation of leukocyte migration. According to their homology, interferons are subdivided into 3 categories. IFN $\gamma$  is a sole member of the type II family. In contrast to the type I family, IFN $\gamma$  is produced primarily by activated subsets of T cells and NK cells, and also NKT cells, macrophages and DCs [38-42]. Canonical activity of IFN $\gamma$  is mediated through the JAK/STAT pathway (Fig. 1-3).



**Fig. 1-3. JAK/STAT pathway.** Binding of IFN $\gamma$  to its receptor triggers oligomerisation of the IFNGR1 and IFNGR2 receptor. Activated JAK kinases phosphorylate cytoplasmic domains of the receptor, enabling association of the STAT1. STAT1 is phosphorylated and after dissociation forms a stable homodimer in a parallel conformational state that migrate to the nucleus and activates transcription by binding to the DNA motifs (e.g. GAS). Conformational change of the STAT1 exposes phosphorylated tyrosine residues and thus facilitates action of phosphatases such as TCP45. Dephosphorylated STAT1 migrates to the cytoplasm where it can be again activated by JAK kinases. Literature data indicates presence of some other posttranslational modifications such as acetylation (Ac), deacetylation (HDAC) and SUMOylation of STAT1.

Binding of IFN $\gamma$  to its receptor triggers oligomerisation of the IFNGR1 and IFNGR2 receptor. These conformational changes bring pre-associated JAK1 and JAK2 kinases into close proximity and facilitate transphosphorylation. Activated JAK kinases phosphorylate cytoplasmatic domains of the receptor, which serve as docking sites for the signal transducer and activator of transcription (STAT1).

STAT1 belongs to a family of transcription factors that consists of seven members with conserved structural similarity [43, 44] (Fig. 1-4).



**Fig. 1-4. Structure of the STAT proteins.** The N-terminal domain (N) is mostly involved in dimer complex formation. The coiled coil domain (CC) facilitates an interaction with transcription factors other than STATs and is involved in nuclear translocation. The DNA binding domain (DNA) promotes binding of STAT to the enhancer element. The linking domain (LK) is necessary for the proper conformation of adjacent domains. SH2 domain mediates binding to the cognate receptor and takes part in the formation of active STAT dimer. Due to the phosphorylation, preserved tyrosine (Y) is exposed and mediates an interaction with the related SH2 domain of the partner STAT. The less conserved domain among STATs is the transcriptional activation domain (T) which recruits transcriptional machinery and regulates gene transcription.

Receptor-bound STAT1 is phosphorylated and after dissociation creates a stable homodimer in a parallel conformational state that translocates to the nucleus and, by binding to the DNA motif, called an interferon-gamma-activated sequence (GAS), activates transcription [45, 46] (Fig. 1-3). Inactivation of STAT1-transcriptional activity is related to conformational change and subsequent dephosphorylation [47, 48]. STAT1 which dissociates from the DNA alters its conformation from a parallel into an antiparallel one. This modification exposes phosphorylated tyrosine residues and thus facilitates action of phosphatases such as TCP45. Dephosphorylated STAT1 migrates to the cytoplasm where it can be again activated by JAK kinases [49, 50]. There is some evidence indicating that in certain conditions the biological

activity of IFN $\gamma$  can be mediated by proteins other than STAT1. However, due to strong affinity of the activated IFNGR1 receptor to STAT1, transcriptional responses to IFN $\gamma$  are dominated by STAT1 [51]. Although tyrosine 701 phosphorylation is crucial for transcriptional activity of STAT1, other studies have indicated the importance of other modifications [52, 53]. It has been shown that serine 727 phosphorylation in the carboxy-terminal domain is necessary for full transcriptional activation of STAT1 [54, 55]. Additionally, other posttranslational modifications such as acetylation and SUMOylation of STAT1 also play a role in regulating its activity. However, in contrast to phosphorylation, these modifications seem to inhibit the transcriptional activity of STAT1 [56-60].

Among the cardiovascular disorders, the role of IFN $\gamma$  is best characterized in atherosclerosis [61]. Most research points to the proinflammatory role of cytokines manifested by involvement of IFN $\gamma$  in the development and progression of atheroma; for example, IFN $\gamma$  was found to be expressed in human lesions and in T cells cloned from human plaques [62]. It was shown that atheroma formation is markedly reduced in genetic knockouts of IFN $\gamma$  [63-66]. Russell et al. showed that a monoclonal antibody to IFN $\gamma$  strikingly inhibited formation of obstructive vascular lesions [67]. Furthermore, Tellides et al. showed that the immunomodulatory effect of IFN $\gamma$  on media expansion was present in the absence of leukocytes, further proving critical role of IFN $\gamma$  in atherogenesis and modelling of cell behavior and cell-cell interactions of all cell types existing in the vessel wall [68]. In addition, a number of research studies have indicated that IFN $\gamma$  boosts macrophage and SMC foam cell formation and inhibits SMC proliferation [61, 69]. Interestingly, other reports suggested that IFN $\gamma$  stimulates proliferation of VSMCs [70, 71]. The function of IFN $\gamma$  in the pathology of atherosclerosis also includes activation and differentiation of T cells as well as stimulation of macrophages in order to express TNF $\alpha$ , IL6 and nitric oxide [61].

Recent studies indicate this cytokine's role in other CVDs. Most of the animal models suggest an important role of IFN $\gamma$  in inflammatory cell recruitment, cytokine and chemokine production, and development of heart failure [72]. Expression of IFN $\gamma$  was highly upregulated in an Ang II-induced model of hypertension [31], and IFN $\gamma$ -deficient mice had reduced heart infiltration by macrophages, which was associated with decreased fibrosis [73, 74]. Additionally, transgenic mice with constitutive expression of IFN $\gamma$  spontaneously developed

myocarditis characterized by inflammation, fibrosis, ventricular wall thinning and dilation as well as reduced systolic function [72, 75]. Clinical data are in line with these animal models and suggest a positive association between IFN $\gamma$  and disease development [76, 77].

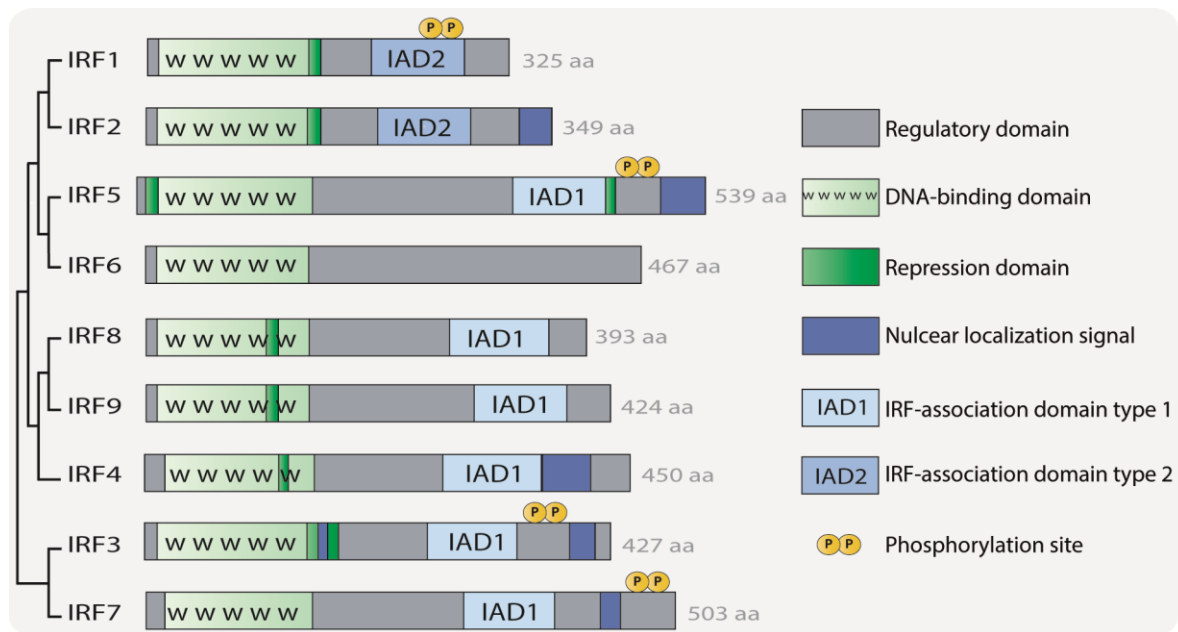
In contrast, other reports revealed the protective effect of IFN $\gamma$ . Garcia et al. showed that IFN $\gamma$ -deficient mice have greater heart hypertrophy as compared to wild-type (WT) animals upon aldosterone infusion [78]. Furthermore, administration of IFN $\gamma$  attenuated myocardial hypertrophy in the rat aortic banding model of pressure overload. Marko et al. demonstrated that in spite of reduced interstitial fibrosis, IFN $\gamma^{-/-}$  mice have more pronounced podocyte injury in the Ang II-induced model of hypertensive organ damage [74]. Not only IFN $\gamma$  but also STAT1 was found to be involved in pathophysiology of CVDs. Agrawal et al. identified STAT1 as an important regulator of foam cell formation and atherosclerotic lesion development [79]. STAT1 was identified to play a role in macrophage apoptosis, a critical process for the formation of necrotic core in atherosclerotic plaques [80]. Mice transplanted with STAT1 deficient bone marrow revealed reduced macrophage apoptosis and plaque necrosis [80].

Taken together, these data suggest that IFN $\gamma$  together with downstream activated STAT1 play a role in the cardiovascular system. However, whether this role is detrimental or protective in the development of CVDs is still not fully understood and should thus be elucidated.

## **IFN and IRFs**

Response to IFN $\gamma$  can be divided into two phases. In the early phase, phosphorylated STAT1 activate genes containing the GAS sequence in their promoters (e.g. *Cxcl9*). Among these genes are also interferon regulatory factors (IRFs) [81] (Fig. 1-5).





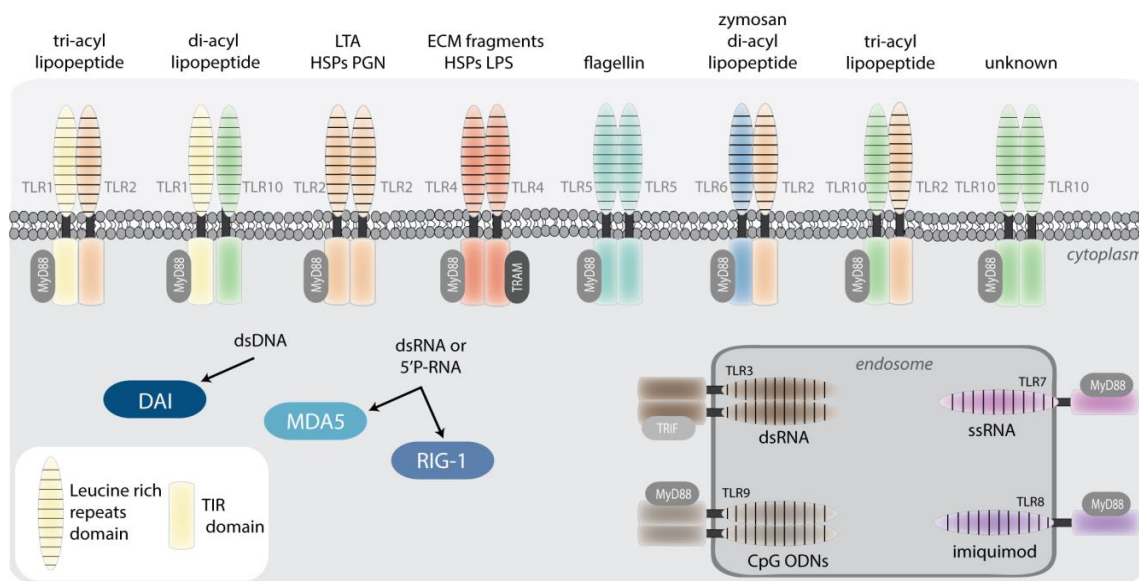
**Fig. 1-5. Phylogenetic relation and structure of the interferon regulatory factors (IRFs).** The N-terminal region of each IRF contains DNA binding domain with five preserved tryptophan residues which form helix-turn-helix structure and recognizes specific DNA sequence e.g. ISRE. Except IRF6, all IRFs contain IRF-association domain (IAD) that allows them to interact with other proteins. Some IRFs contain repression domain and nuclear localization signal domain. Additionally IRF1, IRF2, IRF3 and IRF7 are prone to posttranslational modification e.g. phosphorylation.

These IRFs, by recognizing the modulatory elements, e.g. the interferon stimulated response element (ISRE), trigger the second wave of reaction to the IFN $\gamma$ . The family of IRFs comprises 9 members that share structural similarities [82]. A crucial function of the IRFs in modulation of the transcriptional response is not only based on their ability to directly recognize conserved sequences of target genes, but also on their interaction with other members of the IRF family or other co-factors [83]. IRF3, IRF7, IRF9 play an important role in response to type I interferons. Activated IRF3 and IRF7 induce expression of type I interferons and IRF9 participates in formation of the STAT1-STAT2-IRF9 complex and induces transcription of interferon-stimulated genes [84]. In turn, IRF1 and IRF8 play a particularly important role in IFN $\gamma$  response [51]. Interestingly, recent studies indicate that IRF1 is not only a downstream gene of STAT1 but can also interact with it, thus forming a complex that affects expression of interferon-stimulated genes [85]. Moreover, Wessely et al. revealed an important role of IRF1 in neointimal growth after vessel injury and suggested IRF1 as a target for interventions to prevent hyperplasia [86]. Unlike STAT1 and IRF1, which are ubiquitously expressed, IRF8 expression is thought to be restricted to lymphoid-cell lineages such as B, T and dendritic

cells and macrophages. IRF8 can not only recognize the ISRE element but also, together with other transcription factors including PU.1, it can bind to elements recognized by STAT1 (the GAS element) [87]. Thus, IRF8 may create a feedback loop for some STAT1-activated genes and partially account for the “immune cell-specific” STAT1-dependent functions of IFN $\gamma$  [87]. Interestingly, recently we obtained evidence that IRF8 is highly expressed in ECs and VSMCs after IFN $\gamma$  treatment (Chapter 3), thus suggesting that it can also regulate “vasculo-specific” STAT1-dependent functions of IFN $\gamma$ . Moreover, recent data indicate the function of IRF8 in pathological cardiac hypertrophy or atherosclerosis [88, 89]. Although the mechanism is not clear, it is tempting to speculate that IRF8 specifically regulates STAT1-dependent IFN $\gamma$ -directed transcriptional responses in cell types involved in the vascular function.

## TLRs signaling

Toll-like receptors (TLRs) belong to the group of PRRs that play a pivotal role in the first line of defense against pathogens. Until now, 10 TLRs have been identified in humans (Fig. 1-6).



**Fig. 1-6. Pathogen recognition receptors (PRRs) and their ligands.** Most of the PRRs are located on the cell surface but some receptors are also present in cytosol and endosome. PRRs are activated by multiple PAMPs and thus create first line of defense against infection. All TLRs and their adapters contain highly conservative Toll/IL-1 domain. The adapter protein myeloid differentiation primary response protein-88 (MyD88) seems to be involved in signaling by all TLRs, but not TLR3. TLR4, as the only member of the family, utilizes all four described TIR-containing adapters.

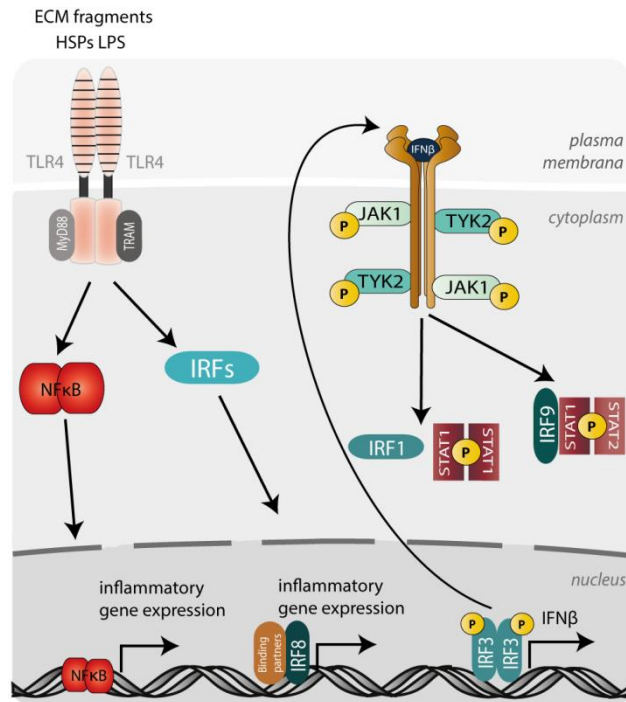
They are expressed on a variety of cell types and play a distinct function in immune recognition [90]. In addition to multiple exogenous PAMPs, TLRs are activated by various

endogenous DAMPs; for example, bacterial lipoproteins and lipoteichoic acid are recognized by TLR2, double-stranded RNA by TLR3 and LPS by TLR4 and TLR2. Likewise, TLR4 recognizes DAMPs related to stress or injury of the host, which include heat shock proteins (HSP), fibrinogen, extra domain A of fibronectin and soluble hyaluronan [91]. All TLRs and their adapters contain highly conservative Toll/IL-1 domain [92, 93]. The adapter protein myeloid differentiation primary response protein-88 (MyD88) seems to be involved in signaling by all TLRs, but not TLR3 [94]. TLR4, as the only member of the family, utilizes all four of the described TIR-containing adapters. TLR4 together with MD2, CD14 and LBP form a complex that recognizes LPS [95]. Response to LPS can be divided into two stages: binding of LPS to the receptor complex located on the cell surface initiates the early phase of activation of the nuclear factor-kappa B (NFkB) through TIR domain-containing adaptors TIRAP (Mal) and MyD88. Subsequently, the TLR4-MD2-LPS complex is internalized to the endosome and used by TRIF and TRAM to activate NFkB. Interestingly, both phases of the response are necessary to activate NFkB [95]. Together this leads to the induction of various target genes that include type I IFNs (through IRF3), chemokines and cell surface molecules.

There is a large number of research studies indicating the importance of the TLR4 pathway in the cardiovascular field [96]. Michelsen et al. showed that mice lacking either TLR4 or MyD88 had reduced atherosclerosis correlated with reduced levels of inflammatory cytokines [97]. TLR4 was found to be overexpressed in the VSMCs of atherosclerotic arteries, even in regions with few inflammatory cells [98]. Other studies pointed to the role of TLR4 in intimal foam cell accumulation [99]. Expression of TLR4 was upregulated in patients with unstable angina and acute myocardial infarction [100]. Recent studies support the role of TLR4 in hypertension. An elevated level of TLR4 was found in spontaneously hypertensive (SHR) rats as well as in the L-NAME-induced model of hypertension and blocking of TLR4 reduced blood pressure, inflammation and maximal mesenteric artery contractile response to noradrenaline [29, 101, 102]. Furthermore, experiments performed in our laboratory provided evidence for the direct role of TLR4 on vascular contractility and blood pressure [103]. The blood pressure of TLR4-deficient mice was not increased upon treatment with L-NAME. This effect was associated by decreased production of reactive oxygen species (ROS) which are known to affect the contraction apparatus of the vessel [104, 105].

## STAT1 and IRFs involved in TLR signaling

As is shown on Figure 1-7, stimulation of TLR4 leads to the phosphorylation of IRF3 and consequently to expression of IFN $\beta$  (type I IFN).



**Fig. 1-7. Components of the JAK/STAT pathway are involved in a TLR signaling.** Stimulation of TLR4 allows TIR domain to interact with accessory molecules which leads to the activation of Myd88 and TRAM. Myd88 activates NFκB, which induces expression of inflammatory cytokines. TRAM activates IRFs e.g. IRF3, IRF8. IRF3 is phosphorylated, homodimerizes and induces IFN $\beta$  production, which in an auto- and paracrine manner can stimulate cells to induce IRF1 and STAT1 in the JAK/STAT dependent manner. Other IRFs e.g. IRF8 during TLR activation interacts with other transcription factors leading to inflammatory gene expression.

IFN $\beta$  in the autocrine/paracrine loop bind to its receptor and activate the pathway. Similarly to IFN $\gamma$ , conformational changes of the receptor facilitate recruitment of the JAK1 and TYK2 kinases which enable formation of predominantly STAT1-STAT2 heterodimers and STAT1 homodimers. Complexes containing STAT1 are transferred to the nucleus. Then the STAT1-STAT2 heterodimers recruit IRF9 (called ISGF3) that bind to promoter regions of genes containing ISRE, whereas STAT1-STAT1 homodimers bind to GAS elements [43, 106].

It is worth noting that both type I and type II IFN (IFN $\gamma$ ) pathways share common features. These two pathways utilize similar transcription factors, e.g. STAT1, IRF1 or IRF8, and transcription factor complexes, and as such regulate partially overlapping genes [107]. Many of the genes (including *Cxcl9*, *Ccl2*, *Ccl5*, *Isg15* and *Nos2*) activated by the TLR pathway are regulated secondary to LPS-induced type I IFN in a STAT1-dependent manner [108]. Activation of IFN is essential to develop a full transcriptional response to TLR4 stimulation; for example, macrophages from Tyk2-deficient mice fail to produce nitric oxide (NO) following

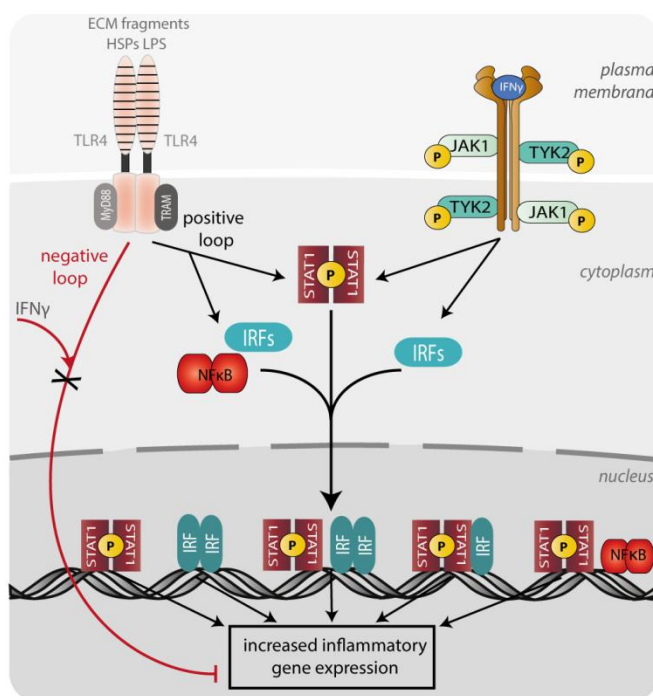
LPS stimulation [109]. As such, STAT1 has been identified as an important mediator in the biological response to TLRs, including TLR4. These studies were further supported by the observation that Socs1 (negative regulator of STAT1 action)-deficient macrophages have increased sensitivity to TLR4 ligands such as LPS and palmitic acid [110]. In addition to IRF3, IRF1, IRF5, IRF7 and IRF8 were shown to contribute to TLR-mediated signaling [82]. Direct interaction of IRF1, IRF5 and IRF7 with MyD88 allows for their activation and subsequent translocation to the nucleus, where they can induce gene expression [111-113]. IRF8-deficient mice fail to induce TLR9-mediated expression of IL6 and TNF $\alpha$  [114]. IRF8 also facilitates TLR2- and TLR4-mediated induction of interleukins, NO synthase and TNF $\alpha$  [115]. Moreover, macrophages from IRF8<sup>-/-</sup> mice produce diminished levels of TNF $\alpha$ , IL1 $\beta$  and IL12p70 in response to LPS [116].

### **Crosstalk between IFN $\gamma$ and TLR**

In physiological conditions the action of immune cells is regulated by the activity of many stimuli. Exposure to one cytokine followed by stimulation with the same or different stimuli may cause either synergistic or antagonistic effects [117]. A similar situation occurs with respect to IFN $\gamma$ , whose pleiotropic action cannot be explained only by the direct function of STAT1 on target genes [118]. Crosstalk between IFN $\gamma$  and TLRs has been associated with host defense against pathogens and injury, but can also contribute to pathophysiology of chronic inflammatory diseases such as rheumatoid arthritis [119]. Indeed, stimulation of DCs and macrophages with IFN $\gamma$  is required to enhance TLR signaling and thus efficient induction of inflammatory mediators [120, 121]. Moreover, it has been shown that IFN $\gamma$  breaks tolerance toward endotoxins (the ligands of TLRs) and increases expression of proinflammatory genes [122]. There are several proposed mechanisms describing functional cooperation between IFN $\gamma$  and TLRs. First, IFN $\gamma$  not only upregulates expression of genes related to TLR signaling such as receptors or genes that participate in signal transduction [120, 123, 124], but also inhibits the negative feedback loop by abrogation of IL10 expression as well as the transcriptional repressors Hes1 and Hey1 [118]. IL10 is an important anti-inflammatory mediator induced by TLR4 to inhibit the inflammatory actions of genes such as TNF $\alpha$  [125]. IFN $\gamma$  increases the activity of serine/tyrosine kinase GSK3 $\beta$  which

in turn inhibits the action of AP-1 and CREB. These two transcription factors are mandatory in order to induce expression of the *IL10* gene, thus inhibition of their action ameliorates the expression of IL10 [126]. Furthermore, IFN $\gamma$  suppress the action of Hes1 and Hey1 repressors and thus augments expression of genes related to inflammation, e.g. IL6 and IL12 [127].

IFN $\gamma$  and STAT1 not only inhibit the negative feedback loop but also enhance positive signaling. Since the discovery that STAT1 can be phosphorylated upon LPS stimulation, the role of STAT1 as an important mediator of the crosstalk between IFN $\gamma$  and TLR4 has been appreciated. Increased phosphorylation of STAT1 and cooperation of STAT1 with other transcription factors may play a role in this amplification mechanism (Fig. 1-8).



**Fig. 1-8. Functional crosstalk between JAK/STAT and TLR pathway.** IFN $\gamma$  may inhibit the negative feedback loop (details in text). JAK/STAT and TLR4 pathway use STAT1 to elicit cell response. Increased phosphorylation of STAT1 upon treatment with IFN $\gamma$  and LPS together with cooperation of STAT1 with other transcription factors like NF $\kappa$ B or IRFs play an important role in the amplification mechanism in immune cells.

Indeed, it has been shown that the activity of STAT1 is enhanced by TLR-dependent induction and the subsequent autocrine activities of IFN $\gamma$  [108, 128, 129]. In contrast, stimulation of macrophages with another TLR ligand, CpG DNA, did not affect phosphorylation of STAT1 and as a consequence did not influence synergistic amplification of the inflammatory genes. Because CpG stimulation does not affect expression of type I IFN, this experiment confirms the importance of the type I IFN autocrine loop in the crosstalk between IFN $\gamma$  and TLR4 [130]. Other studies indicate that STAT1 cooperation with other transcription factors such as NF $\kappa$ B

at the level of target gene promoters is mandatory for the synergistic amplification of inflammatory genes [120].

These phenomena were observed for example for an expression of chemokines such as *Cxcl9*, *Cxcl10* adhesion molecule *Icam1* or *Nos2* in response to IFN $\gamma$  and LPS or other inflammatory mediators [131-137]. In addition, STAT1 targets IRF1 and IRF8 have also been shown to contribute to signal integration between IFN $\gamma$  and LPS. Sequences recognized by both STAT1 and NF $\kappa$ B were found in the promoter regions of IRF1, thus indicating that not only IFN $\gamma$  but also TLR agonists can regulate expression of IRF1 [138-141]. Similarly, cooperation between IRF1 and NF $\kappa$ B was found to be involved in the transcriptional regulation of *Cxcl10* and *Nos2* (*iNOS*) [142, 143]. Moreover, other reports have suggested the role of IRF8 in IFN $\gamma$ - and LPS-mediated synergistic induction of pro-inflammatory genes such as *Il1*, *Il6*, *Il12* and *Tnfa* as well as the chemokine *Ccl5* (*RANTES*) [115, 144]. These findings suggest that in immune cells, STAT1 and IRFs together with NF $\kappa$ B coordinate antimicrobial and inflammatory synergism between IFN $\gamma$  and TLRs.

Recently, a new mechanism of signal integration between IFN $\gamma$  and TLR4 has been proposed [85]. This mechanism is based on epigenetic changes triggered by IFN $\gamma$  which augments expression of TLR4 downstream genes. Qiao et al. suggested that IFN $\gamma$ -activated STAT1 affects histone acetylation and thus causes increased and prolonged recruitment of additional transcription factors and pol II after TLR4 stimulation. Consequently this mechanism increases transcription of proinflammatory genes. As such, STAT1 may not only be considered as a transcription factor but also as an element that initiates chromatin remodeling.

Most studies performed so far have indicated the importance of signal integration between JAK/STAT and TLR4 pathways in immune cells. However, our knowledge about this functional cooperation in non-immune cells is limited.

## Goals /Scope of the thesis

We hypothesized that STAT1- and IRF-mediated gene expression accelerates an inflammatory response, which negatively affects the cardiovascular system.

In **Chapter 2** we introduce the concept of signal integration in non-immune cells represented by ECs, VSMCs and proximal tubular cells. Data presented in this chapter provide evidence for crosstalk between IFN $\gamma$  and LPS. Increased activity of STAT1 and IRF1 resulted in amplified expression of proinflammatory cytokines *Cxcl10*, *Cxcl9*, *Ccl5* and an inducible nitric oxide producer – *Nos2* (iNOS). Thus we consider STAT1 as a novel target for therapeutic intervention also in non-immune cells.

In **Chapter 3** we elucidate the role of STAT1 and IRF8 in mediating the interplay between a damaged organ and host immunity. In this chapter we present the results of a genome-wide analysis in VSMCs which identified a set of STAT1-dependent genes that were synergistically affected by interactions between IFN $\gamma$  and TLR4. Among the highly amplified genes we distinguished not only chemokines, adhesion molecules, antiviral and antibacterial genes, but also the gene encoding *Irf8*, the transcription factor that was not known to be expressed in the vasculature. We identify *Ccl5* and *Nos2* as the potential targets of *Irf8*. Finally, the functional assays together with the immunohistochemical stainings of phosphorylated STAT1- and STAT1-dependent genes presented in this chapter support the importance of STAT1 in the regulation of vascular inflammation.

Data presented in **Chapter 4** disclose the role of STAT1 as an important regulator of inflammation and vessel function in the model of Ang II-induced hypertensive end organ damage. Compared with the control, STAT1-deficient animals infused with Ang II had ameliorated immune cell infiltration of the heart and kidney, reduced fibrosis and, foremost, improved vessel function. We identified several STAT1-dependent genes that may participate in the progression of vascular damage and thus contribute to progression of the disease. Among them, *Cxcl10*, *Ccl2* and *Cxcl10* chemokines and proteins involved in regulating oxidative stress (*Nox4*, *p47phox*, *p22phox*) revealed to be regulated by STAT1. Interestingly, despite diminished CD45<sup>+</sup> cell infiltration and expression of fibrotic markers, STAT1<sup>-/-</sup> animals as compared to wild type (WT) animals had a significantly higher concentration of urinary



albumin, thus indicating increased glomerular damage. We hypothesize disturbance of autophagy to be a cause of albuminuria in STAT<sup>-/-</sup> and suggest a novel role of STAT1 in response to stress in the kidney.

**Chapter 5** summarizes findings presented in the thesis and discuss potential applications as well as future research directions.

## **Chapter 2**

### **STAT1-dependent signal integration between IFN $\gamma$ and TLR4 in non-immune cells**

#### **Introduction**

Cell signaling is a complex system that facilitates perception and reaction to stimuli. Proper processing of the signaling is mandatory for the functioning of cells, tissue homeostasis and, consequently, survival of the organism as a whole. Many diseases are related to improper response to intra- or extracellular ligands. Until very recently scientists studied linear signaling cascades; however, because cells have to integrate multiple signals in order to regulate manifold cellular processes, it became clear that there must be crosstalk between them. Inflammation is a sophisticated mechanism of response to an infectious agent and injury [145]. This mechanism is based on a complex cell signaling network that maintains homeostasis of the host. However, in certain conditions the system that prevents injury may contribute to its progression. Excessive inflammation is involved in the pathophysiology of many diseases, including atherosclerosis, aortic aneurysm formation or acute kidney injury. One of the essential contributors of inflammation is IFN $\gamma$ , which is produced mostly by T and NK cells [38-42]. IFN $\gamma$  signaling plays an important role in innate and adaptive immunity by activating immune cells such as macrophages or T cells. Recent evidence have indicated the significance of IFN $\gamma$  signaling in non-immune cells; for example, in the absence of immune cells, IFN $\gamma$  can cause proliferation of SMCs in the media layer of the vessel wall [68]. IFN $\gamma$ -dependent chemokines, such as Cxcl10 (IFN-induced protein of 10 kDa, or IP10) or Cxcl9 (a monokine induced by IFN $\gamma$  or MIG), are highly expressed upon stimulation in endothelial cells (ECs) and vascular smooth muscle cells (VSMCs), and are present in dysfunctional vessels [146-149]. Other studies revealed that IFN $\gamma$  deficient mice demonstrated decreased tubulointerstitial damage upon treatment with Angiotensin [74]. The signal transducer and activator of transcription (STAT)-1 is a canonical mediator of IFN $\gamma$  signaling. Activation of STAT1 by IFN $\gamma$  leads to its phosphorylation and formation of the dimer that triggers expression of STAT1-dependent genes. Importantly, recent experiments performed in macrophages have shown that STAT1 is not only involved in the activation of the JAK/STAT pathway, but also contributes to signaling events mediated by Toll-like receptor 4 (TLR4) [108, 150].

TLR4 belongs to the receptor family that recognizes pathogen-associated molecular patterns such as lipopolysaccharide (LPS) and activates expression of proinflammatory genes. In the second stage of the response to TLR4 stimulation, TIR-domain-containing adapter-inducing interferon- $\beta$  (TRIF) triggers phosphorylation of transcription factor IRF3 and consequently expression of IFN $\beta$  which, in turn, facilitates activation of STAT1 [151]. Moreover, IFN $\gamma$  can sensitize immune cells to the action of LPS, which greatly amplifies the inflammatory response [152]. Thus STAT1 may be considered as an important point of crosstalk between LPS and IFN $\gamma$  signaling in macrophages [120].

In this chapter we provide evidence to support the idea that in addition to the immune system, signal integration between these two pathways is present in other tissue types. IFN $\gamma$ -mediated activation of STAT1 serves as a platform for increased LPS signaling, resulting in augmented STAT1 phosphorylation and expression of genes related to chemotaxis and oxidative stress. As evidence for this concept we use inhibitors of the JAK/STAT pathway that are known to affect directly (stattic) or indirectly (Ag490 – JAK2 inhibitor) the action of STAT1.

## **Material and Methods**

### **Cell culture**

#### *HMECs*

Human Microvascular Endothelial Cells (HMECs) obtained from Centers for disease control and prevention (Atlanta, GA, USA) were cultivated in MCDB-131 (Life Technologies) medium containing 10% FBS (PAA), 100U/ml penicillin, 100 $\mu$ g/ml streptomycin, 0.01 $\mu$ g/ml EGF, 0.05 $\mu$ M hydrocortisone (Sigma), 2mM L-glutamine (PAA).

#### *Isolation of primary VSMC*

WT mice (strain background *C57BL/6*) were obtained from Charles River Laboratories (Sulzfeld, Germany). Primary Vascular Smooth Muscle cells (VSMCs) were isolated by enzymatic digestion [153] in a solution containing collagenase type II 1mg/ml, soybean trypsin inhibitor 1mg/ml (Life Technologies), elastase 0.744u/ml (Sigma) in HBSS (Life Technologies). Isolated aortas from 2 mice were cleaned from perivascular fat and predigested for 10min. Subsequently adventitia was removed, aortas were cut lengthwise and intima was removed

by gentle scraping. So prepared aortas were enzymatically digested for 1h at 37°C. After digestion aortas were passed through 100µm cell strainer (BD Falcon) and left undisturbed on 3 wells of a 48 well plate for 1 week. Until passage number 3 cells were cultivated in DMEM (PAA) medium with 20% FBS. After 3<sup>rd</sup> passage SMC were cultivated in DMEM (PAA) medium containing 4.5mg/l Glucose, 2mM L-glu, supplemented with 100U/ml penicillin and 100µg/ml streptomycin and 10% FBS (PAA). Homogeneity of the culture was assessed by the expression of  $\alpha$ -smooth muscle actin, calponin and smoothelin.

#### *Isolation of tubular cells*

Freshly isolated kidneys were minced and placed in a HBSS solution containing collagenase II, 1mM HEPES and 100U/ml penicillin, 100µg/ml streptomycin (Life Technologies). After 1h incubation at 37°C in water bath, solution were sieved over a 70µm cell strainer and centrifuged. Subsequently cells were washed and resuspended in the DMEM/F12 medium containing GlutaMAX (Life Technologies) with 5% FBS (Sigma), 25mM HEPES, 100U/ml penicillin, 100µg/ml streptomycin (Life Technologies), 25ng/ml epidermal growth factor (Sigma) and 5ml of hormone mixture containing Insulin (0.5mg/ml), PGE<sub>1</sub> (0.125µg/ml), Triiodothyronine (3.38ng/ml), Hydrocortisone (1.8µg/ml), Transferrin (0.173µg/ml) and Sodium Selenite (0.5mg/ml) (Sigma). Cell were placed on a collagen covered plate and used for the experiments after second splitting. Homogeneity of the culture was assessed by the expression of sodium/potassium-transporting ATPase gamma chain (FXD2).

#### *Isolation of splenocytes*

Freshly isolated spleens were placed in the RPMI 1640 (Life Technologies) medium containing 25mM Hepes (Gibco), 1% FBS (Sigma), 2mM L-glutamine, 100U/ml penicillin, 100µg/ml streptomycin (PAA). Afterwards, spleens were minced with scalpel and passed through pre-wetted 70µm and 40µm cell strainers (BD Biosciences). To lyse red blood cells, cell suspension was centrifuged and resuspended in RBC lysis buffer (eBiosciences). After 45sec of incubation medium was added and cells suspension was centrifuged. Next, cells were counted and treated with indicated concentration of either LPS or IFN $\gamma$  or both.

In general, all cells were treated with 10ng/ml of IFN $\gamma$  and/or 1 $\mu$ g/ml of LPS. Treatment of VSMCs and HMECs was performed in medium containing 2% (splenocytes in 1%) serum without the addition of growth factors (starving medium), after starvation of at least 12h before an experiment. Treatment of proximal tubular cells was performed in regular medium after refreshment.

### **Western analysis**

Protein extracts from cells as well were prepared using RIPA buffer (Sigma) containing protease and phosphatase inhibitors (Roche). Cell lysates were collected and stored at -80°C. Protein concentrations were measured using BCA protein assay (Pierce). Protein extracts were heated with sample buffer (Life Technologies) containing dithiothreitol (90°C, 10min) and loaded on NuPAGE Bis-Tris Gel (Life Technologies). After electrophoresis (200V, 40min to 1h 30min, depending on protein size), proteins were transferred onto PVDV membranes (Millipore) using wet transfer system (Bio-rad, 30V, 90mA, 16h at 4°C). Membranes were blocked either with 5% nonfat dry milk or with 5% BSA in TBS-Tween (TBST) and incubated with primary antibodies: phospho-STAT1 (Tyr 701) antibody (overnight, 1:1000, Cell Signaling, cat no. 3171 ), phospho-STAT1 (Ser 727) (overnight, 1:1000, Cell Signaling, 8826), STAT1 (1h at room temperature, 1:200 Santa Cruz, SC346) or GAPDH (overnight, 1:15000, Cell Signaling, 5174). After washing in TBST, membranes were incubated with secondary goat anti-rabbit antibody (30min 1:10000 for STAT1 and 1:15000 for all the others, Santa Cruz Biotechnology SC2004). Antibody-antigen complexes were visualized using Luminata Forte or Luminata Classico (only for GAPDH) Western HRP substrate (Millipore) in INTAS imaging system (Intas, Germany).

### **Measurement of nitric oxide (NO)**

VSMCs were treated as depicted in cell experiment section. After treatment medium was refreshed and cells were cultivated for further 24h. Subsequently, medium was collected and 100ul was used to measure amount of NO by Griess diazotization reaction [154]. Medium was incubated with freshly prepared solution containing 1% sulfanilamide 5% HCl, 0.1% aqueous solution of 2-(1-Naphthylamino)ethylamine dihydrochloride (Sigma). After 10min incubation OD at 560nm was measured and compared to the standard curve.

## RNA isolation and PCR and real-time PCR

Total RNA was isolated using RNAeasy Mini Kit (Qiagen, Hilden, Germany) together with DNase digestion step according to the manufacture's protocol. Complementary DNA was synthesized using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, USA), according to manufacturer's protocol. Quantitative reverse transcriptase PCR (qRT-PCR) was performed using SSoFast Evagreen (MyiQ ICycler, Bio-Rad). Forward and reverse primers are depicted in Table 2-1. The  $2^{-ddCt}$  method was applied for quantification [155]. Fold change in the target gene were normalized to *GAPDH* and relative to the expression at untreated sample.

**Table 2-1. List of primers used in chapter 2.**

Gene Name	Forward	Reverse
<b>Gapdh</b>	TCGGTGTGAACGGATTTGGC	TTTGGCTCCACCCTTCAAGTG
<b><math>\alpha</math>-actin</b>	CAACTGGTATTGTGCTGGACT	GAAAGATGGCTGGAAGAGAGT
<b>calponin</b>	ACGGCTTGCTGCTGAAGTA	AAGATGTCGTGGGGTTTCAC
<b>smoothelin</b>	AGAACTGGCTACACTCTCAAC	GGGTCCAATGTGTGTGCTG
<b>Ccl5 (Rantes)</b>	CGCACCTGCCTCACCATAT	CACTTCTTCTCTGGGTGGC
<b>Cxcl10</b>	TCATCCCTGCGAGCCTATCC	GGAGCCCTTTTAGACCTTTTT
<b>Cxcl9</b>	CTGCCATGAAGTCCGCTGTTCT	TCCCCCTCTTTTGCTTTTCTT
<b>iNOS (nos2)</b>	TGGGGCAGTGGAGAGATTTT	TCTGGTCAAACCTTGGGGT
<b>FXD2</b>	ATGGCTGGGGAAATATCAGAT	ACCTGCCTATGTTTCTTACCG
<b>IRF1</b>	AAAAGAGCCAGATCCCAAGAC	AACATCTCCACACAGCTTCC
<b>STAT1</b>	AACATACGGAAAAGCAAGCG	GCTGTTCTGTTTTGGTCG
<b>TNF<math>\alpha</math></b>	Qiagen cat no. QT00104006	
<b>Gapdh_human</b>	CAACTGCTTAGCACCCCTGG	CAGGTCAGGTCCACCACTGA
<b>Ccl5 human</b>	CCCTCGCTGTCATCCTCATT	GTGACAAAGACGACTGCTGG
<b>Cxcl10 human</b>	CGTGTGAGATCATTGCTACAA	GACCTTTCCTTGCTAACTGCT
<b>Cxcl9 human</b>	GTGGTGTCTTTTCCTCTTGGG	CTCACTACTGGGGTTCCTTGC

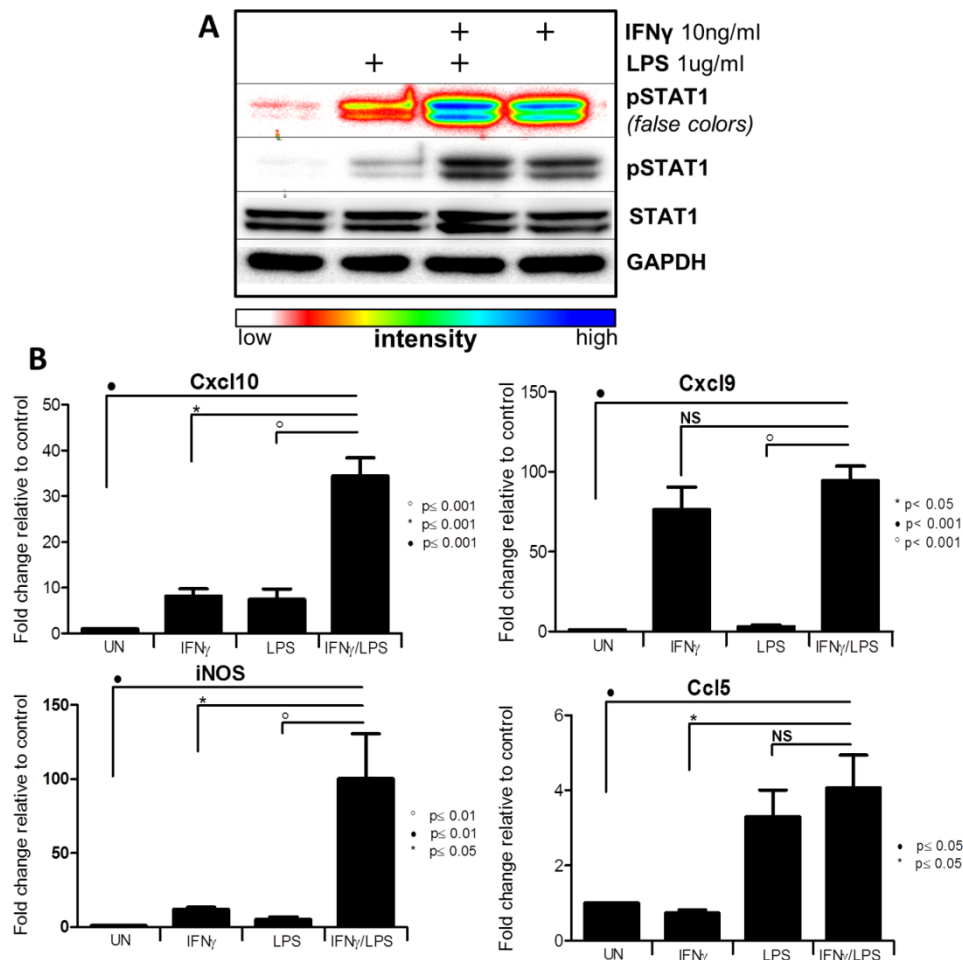
## Data analysis

Statistical analysis: Results are expressed as mean  $\pm$  SEM for at least 3 experiments. Data were compared by a One Way ANOVA and the Tukey post-hoc test or T-test, when appropriate. A probability value  $p < 0.05$  was considered statistically significant. All statistical tests were performed with GraphPad Prism <sup>®</sup> 5.0.

## Results

### IFN $\gamma$ sensitizes splenocytes for LPS-induced STAT1-phosphorylation and target gene expression.

To verify in our laboratory presence of the signal integration between IFN $\gamma$  and LPS in immune cells, we isolated splenocytes from *C57BL/6* mice. Spleen is the largest secondary immune organ that contains mostly T and B cells [156].

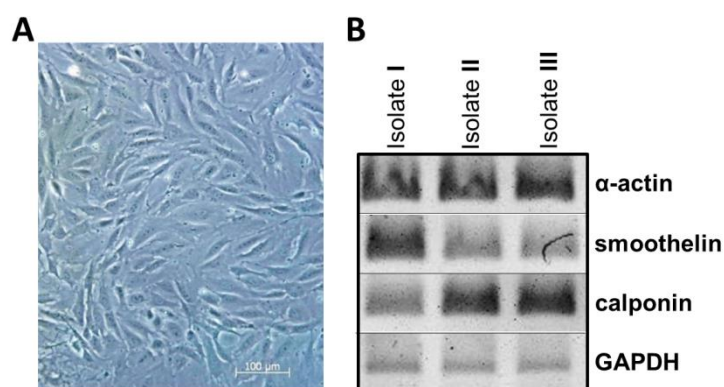


**Fig. 2-1. Signal integration between IFN $\gamma$  and LPS in splenocytes.** Isolated splenocytes from 3 animals were treated with 10ng/ml of IFN $\gamma$  for 8h or 1  $\mu$ g/ml of LPS for 4h, or both. **A**, Protein extracts were analyzed by western blotting for tyrosine phosphorylated STAT1 (pSTAT1), total STAT1 and GAPDH. Beside classical presentation, palette of inverted false colors for pSTAT1 was applied where white indicates low and blue high intensity of the band. **B**, Splenocytes were treated as in A. RNA was isolated and subjected to qRT-PCR for *Cxcl10*, *Cxcl9*, *Ccl5* and *iNOS*.  $p < 0.05$  was considered significant.

Preliminary experiments (verifying different time points and ligand concentrations) performed in our laboratory revealed that the highest level of sensitization for the action of LPS occurs when the cells are treated with 10ng/ml of IFN $\gamma$  for 4h followed by 1 $\mu$ g/ml of LPS for another 4h. Pretreatment of splenocytes with such a conditions (Fig. 2-1 A) resulted in a significant increase in STAT1 phosphorylation as compared to both factors alone. Increased STAT1 expression was also observed and was strictly dependent on IFN $\gamma$  (Fig. 2-1 A). Next, we examined expression of proinflammatory genes, chemokines: *Cxcl10*, *Cxcl9*, *Ccl5* and inducible nitric oxide producer – *Nos2* (iNOS) (Fig. 2-1 B). We identified chemokine *Cxcl10* and *Nos2* as genes that are synergistically amplified upon combined treatment. Both were expressed moderately upon treatment with IFN $\gamma$  or LPS alone and highly amplified in the presence of IFN $\gamma$  and LPS (Fig. 2-1 B, left panel). These results correlated with increased STAT1 phosphorylation (Fig. 2-1 A). On the contrary we could not detect significantly increased mRNA levels of both *Cxcl9* and *Ccl5* treated with IFN $\gamma$  and LPS compared to both factors alone (Fig. 2-1 B, right panel). Interestingly, expression pattern of this two genes were different. *Cxcl9* expression was IFN $\gamma$  dependent and LPS independent whereas *Ccl5* was expressed only upon treatment with LPS.

### **IFN $\gamma$ sensitizes SMCs and ECs for LPS-induced STAT1-phosphorylation and target gene expression.**

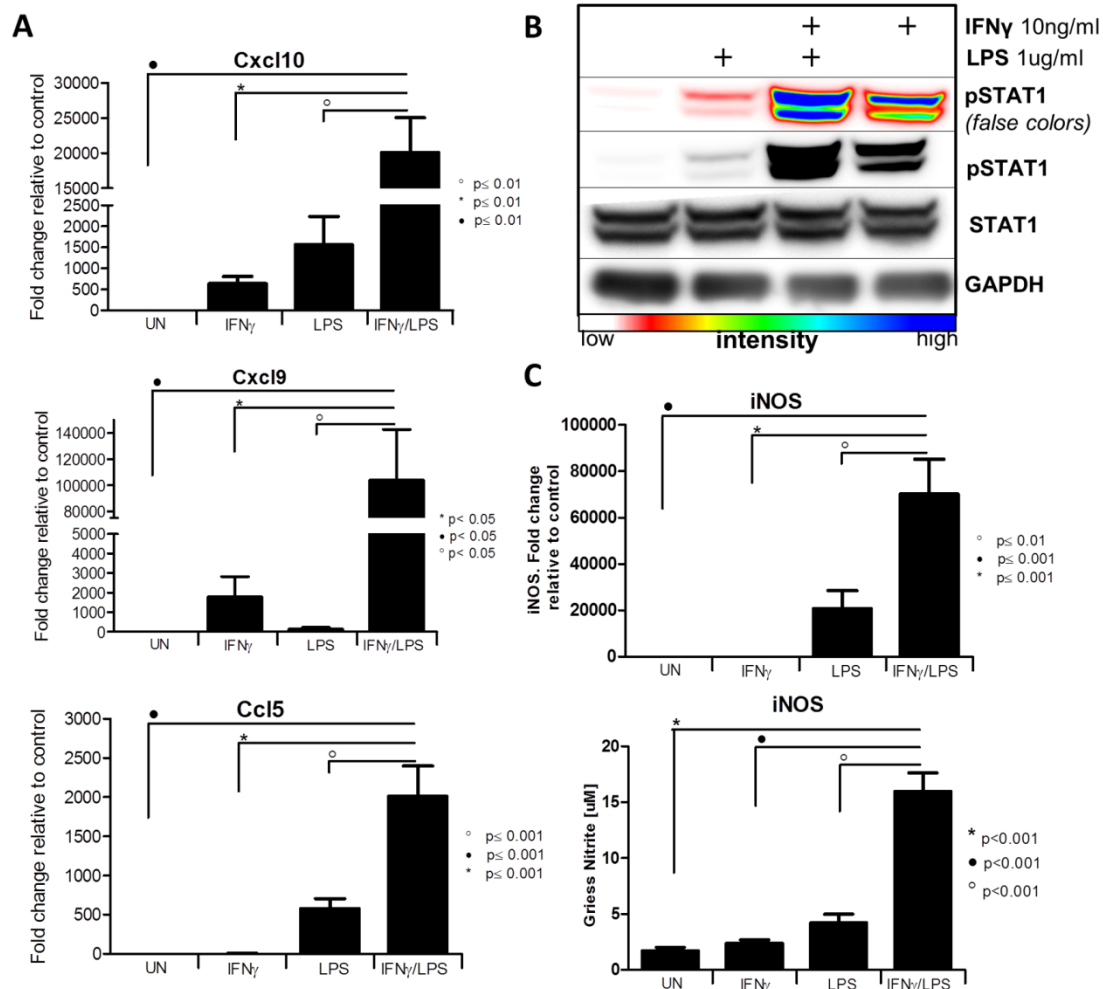
Next, we studied the possibility of STAT1-dependent cross-talk in cells from the vasculature. We isolated primary VSMCs from *C57BL/6* aortas according to the method described in section material and methods. Representative picture of isolated VSMCs is present in Fig. 2-2 A.



**Fig. 2-2. Isolation of aortic VSMCs and assessment of their homogeneity.** Cells were isolated by enzymatic digestion. Representative picture of cultivated VSMCs is given in **A**. **B**, To evaluate homogeneity of the culture, RNA was isolated and subjected to PCR for  $\alpha$ -actin, smoothelin and calponin. All cells expressed markers characteristic for VSMCs.

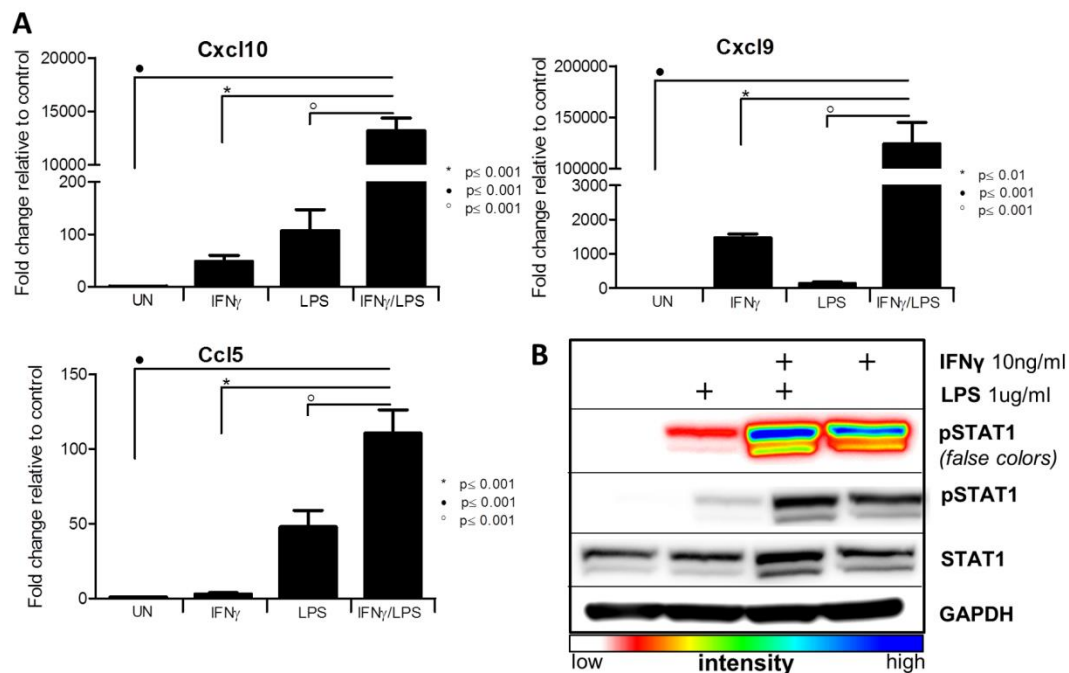


Assessment of the homogeneity was performed using markers specific to VSMCs (Fig. 2-1 B). Indeed, VSMCs expressed  $\alpha$ -actin, smoothelin and calponin that are characteristic for VSMCs [157]. Pretreatment of VSMCs with IFN $\gamma$  for 4h followed by LPS for another 4h resulted in a significant increase in STAT1 phosphorylation as compared to both factors alone (Fig. 2-3 B). Increased levels of phosphorylated STAT1 were correlated with synergistic amplification of chemokines *Cxcl10*, *Cxcl9* and *Ccl5* upon combined treatment (Fig. 2-3 A).



**Fig. 2-3. Signal integration between IFN $\gamma$  and LPS in VSMCs.** A, Isolated primary VSMCs from murine aortas were treated with 10ng/ml of IFN $\gamma$  for 8h or 1  $\mu$ g/ml of LPS for 4h, or both. RNA was isolated and subjected to qRT-PCR for *Cxcl10*, *Cxcl9*, *Ccl5*. B, VSMCs were treated as in A. Expression of *Nos2* upon stimulation (left panel) and the product of *Nos2*- nitrite in the conditioned medium (right panel) was investigated.  $p < 0.05$  was considered significant. C, VSMCs were treated as in A. Protein extracts were analyzed by western blotting for p $_{\text{Ty}}$ STAT1, total STAT1 and GAPDH. Beside classical visualisation, palette of inverted false colors for p $_{\text{Ty}}$ STAT1 was applied where white indicates low and blue high intensity. Representative picture is present.

Interestingly, 3 different chemokines revealed 3 different patterns of expression upon treatment suggesting different mechanism of activation. Cxcl10 (Fig. 2-3 A,) was highly expressed upon treatment with IFN $\gamma$  and LPS alone and combined treatment synergistically amplified this response. Expression of Cxcl9 (Fig. 2-3 A, middle panel) upon treatment with LPS was very low compared to IFN $\gamma$  stimulation and highly amplified in the presence of both. In contrast expression of Ccl5 was very low upon treatment with IFN $\gamma$ , highly expressed with LPS and synergistically amplified in the presence of both stimuli (Fig. 2-3 A). Likewise expression of Nos2 (iNOS) was high upon treatment with LPS and highly amplified upon combined treatment (Fig. 2-3 C). The RNA levels reflected nitrite accumulation for Nos2 (Fig. 2-3 C, lower panel). Because we were not able to isolate a homogeneous population of endothelial cells (data not shown), we instead used the human microvascular endothelial cell line (HMEC). This cell line retains morphologic, phenotypic, and functional characteristics of normal microvascular ECs [158]. Similarly to VSMCs, synergistic expression of chemokines Cxcl10, Cxcl9 and Ccl5 was identified in HMECs treated with IFN $\gamma$  and LPS (Fig. 2-4 A), which coincided with STAT1 phosphorylation (Fig. 2-4 B).



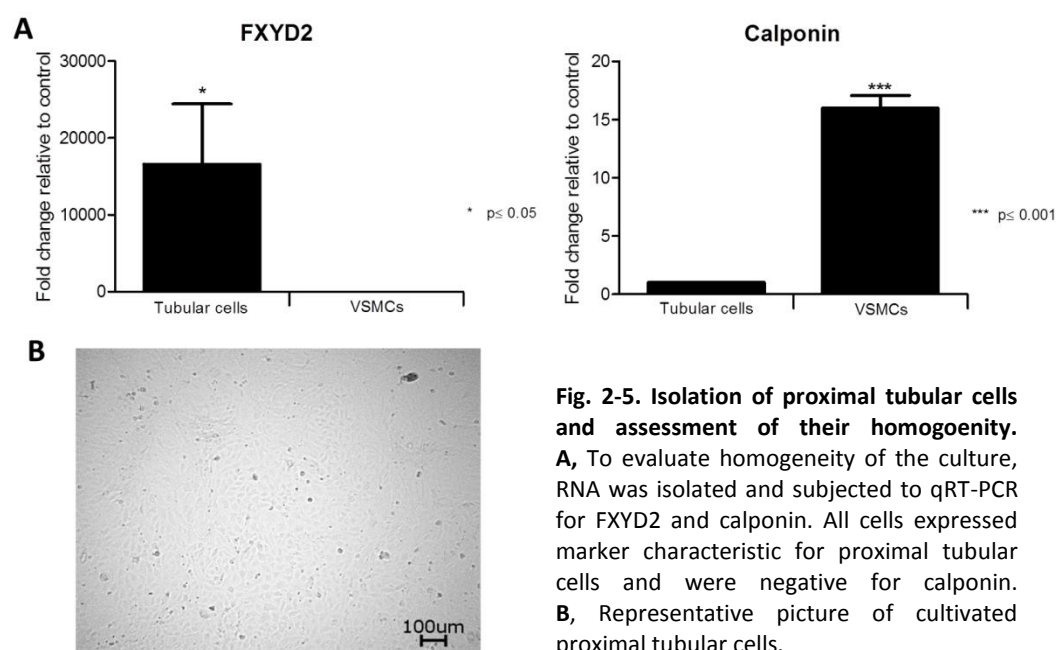
**Fig. 2-4. Signal integration between IFN $\gamma$  and LPS in HMECs.** **A**, Cultivated HMECs were treated with 10ng/ml of IFN $\gamma$  for 8h or 1  $\mu$ g/ml of LPS for 4h, or both. RNA was isolated and subjected to qRT-PCR for *Cxcl10*, *Cxcl9*, *Ccl5*.  $p < 0.05$  was considered significant. **B**, HMECs were treated as in A. Protein extracts were analyzed by western blotting for p<sub>Tyr</sub>STAT1, total STAT1 and GAPDH. Beside classical visualisation, palette of inverted false colors for p<sub>Tyr</sub>STAT1 was applied where white indicates low and blue high intensity. Representative picture is present.

Likewise, expression of the chemokines had the same profile as in VSMCs. Moreover, we observed that pretreatment of HMECs with IFN $\gamma$  for 4h followed by LPS for another 4h resulted in a significant increase in STAT1 phosphorylation as compared to both factors alone (Fig. 2-4 B). Increased STAT1 protein levels, strictly dependent on IFN $\gamma$ , could provide a possible explanation for the increased STAT1 phosphorylation under conditions when both IFN $\gamma$  and LPS are present.

### Signal integration is present in proximal Tubular cells.

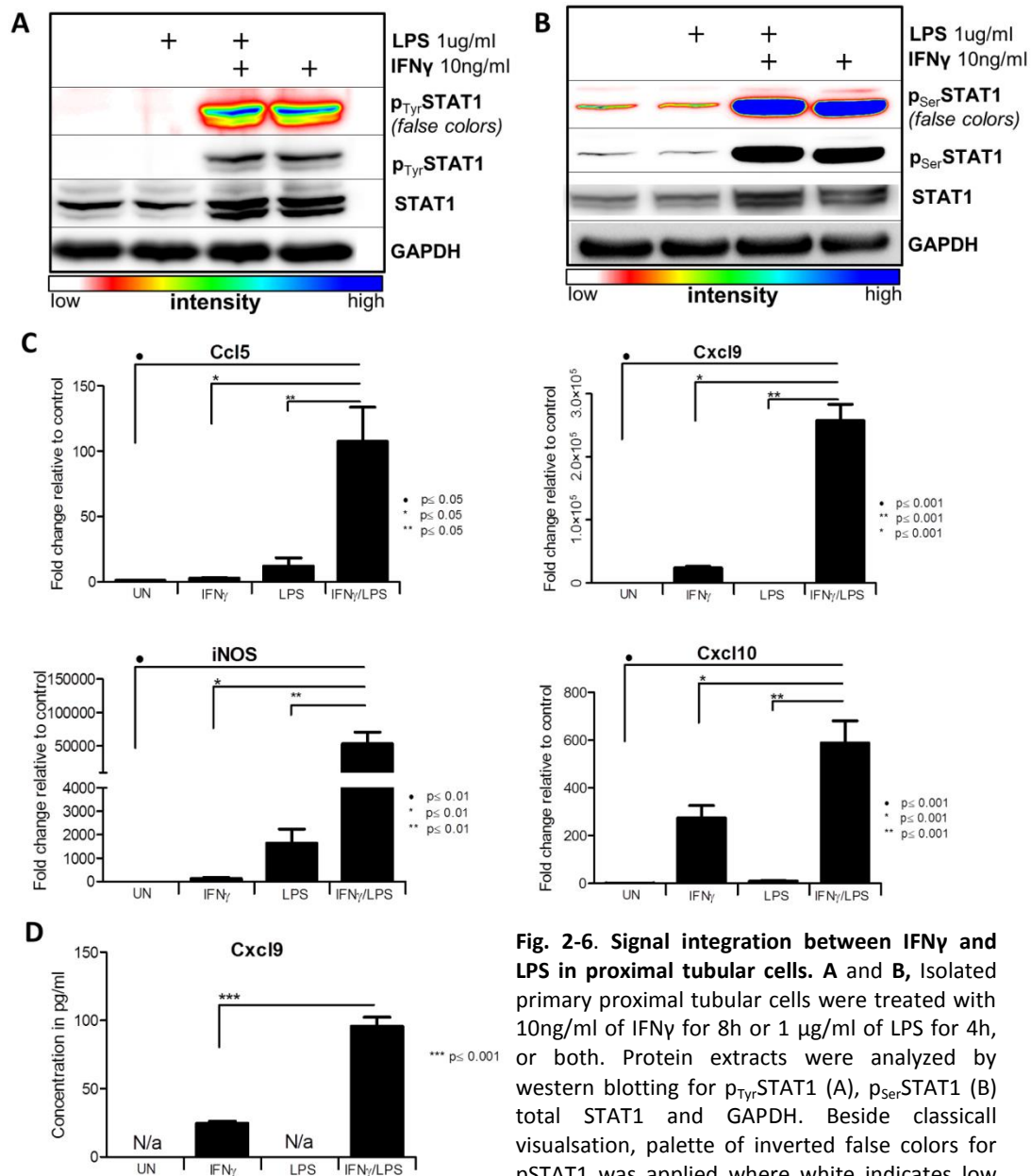
Similarly to cells from the vasculature also tubular epithelial cells are the target of inflammatory response [159]. Thus to support hypothesis that signal integration is present in non-immune cells, we isolated proximal tubular cells from *C57BL/6* mice. Representative picture of tubular cells is present in Fig. 2-5 B Homogeneity of the isolated cells was assessed by expression of FXYD2.

Isolated cells expressed high levels of FXYD2, marker for tubular cells and low levels of calponin which is preferentially expressed in smooth muscle cells (Fig. 2-5) [157].



Next, we analyzed expression and activity of STAT1 protein. Treatment with either IFN $\gamma$  alone or together with LPS revealed upregulation of STAT1 expression (Fig. 2-6 A). In contrast to cell from the vasculature, we could not detect neither increased STAT1 expression upon combined treatment nor phosphorylation upon treatment with LPS alone. Interestingly, also

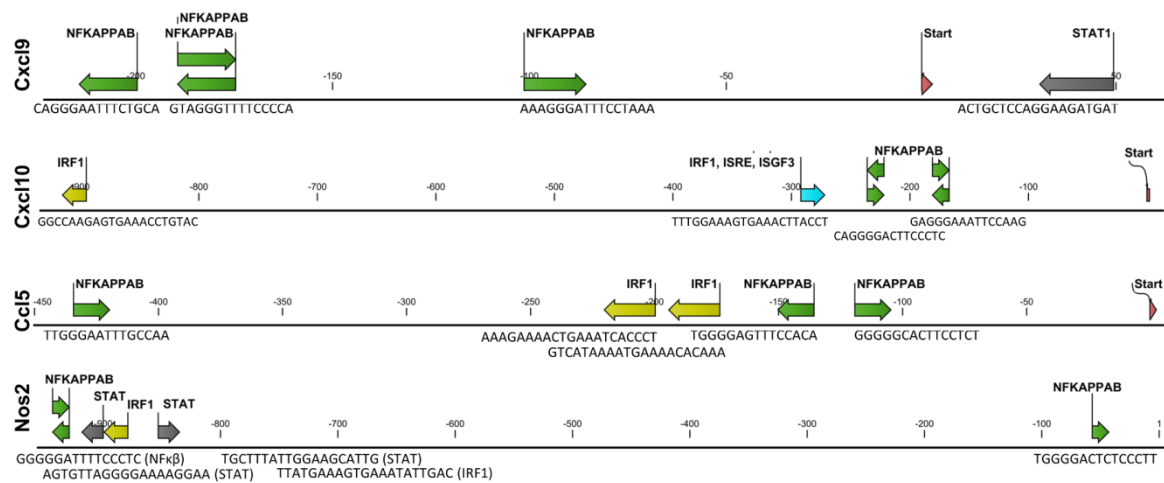
phosphorylated serine was not different between IFN $\gamma$  and IFN $\gamma$  + LPS treated samples (Fig. 2-6 B). Subsequently, we examined expression of chemokines *Cxcl10*, *Cxcl9*, *Ccl5* and *Nos2* (Fig. 2-6 C). All investigated genes were moderately expressed upon treatment with IFN $\gamma$  or LPS and synergistically amplified upon combined treatment. ELISA performed on the medium remained after treatment of proximal tubular cells with IFN $\gamma$  and LPS, confirmed synergistic amplification of Cxcl9 after treatment with both stimuli (Fig. 2-6 D).



**Fig. 2-6. Signal integration between IFN $\gamma$  and LPS in proximal tubular cells.** **A** and **B**, Isolated primary proximal tubular cells were treated with 10ng/ml of IFN $\gamma$  for 8h or 1  $\mu$ g/ml of LPS for 4h, or both. Protein extracts were analyzed by western blotting for p<sub>Tyr</sub>STAT1 (**A**), p<sub>Ser</sub>STAT1 (**B**) total STAT1 and GAPDH. Beside classical visualisation, palette of inverted false colors for pSTAT1 was applied where white indicates low and blue high intensity. Representative pictures are present. **C**, Tubular cells were treated as in **A**. RNA was isolated and subjected to qRT-PCR for *Cxcl10*, *Cxcl9*, *Ccl5* and *iNOS*. **D**, Cells were treated as in **A**. On the medium remained after treatment ELISA for *Cxcl9* was performed. p<0.05 was considered significant. N/a - not detected.

## Promoter analysis of the potential STAT1-targets

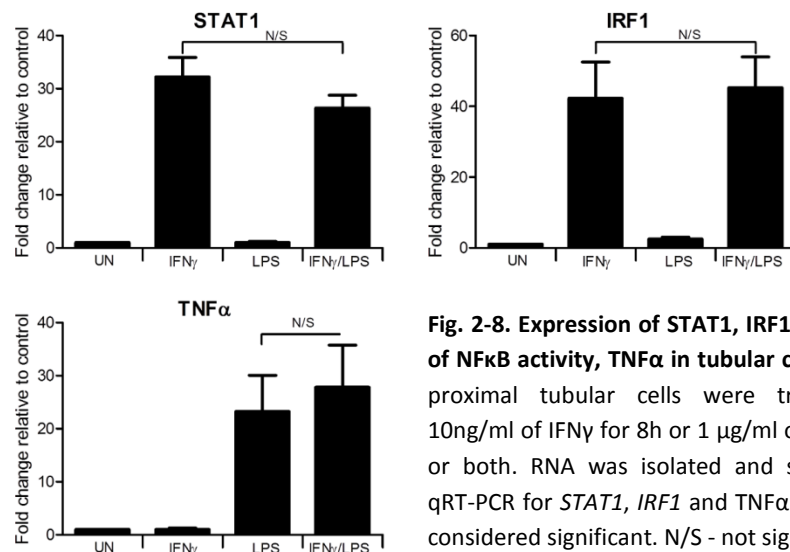
Next, to provide *in silico* evidence for the importance of STAT1 and to locate other transcription factors that potentially may be involved in the synergistic amplification of the gene expression, we searched their promoter regions for overrepresented motifs recognized by transcription factors (Fig. 2-7). Promoter analysis of the synergistically upregulated genes predicted the presence of STAT-NFκB and IRF-NFκB motifs, strongly suggesting the cooperative involvement of NFκB, STAT1 and/or IRFs in the transcriptional regulation of *Cxcl9*, *Cxcl10*, *Ccl5* and *Nos2* in response to IFNγ and LPS.



**Fig. 2-7. Promoter analysis of the *Cxcl9*, *Cxcl10*, *Ccl5*, *Nos2*.** The promoter regions from -1000 to +100bp from transcription start site were searched for binding sites. Promoters for amplified STAT1 dependent genes were screened using GENOMATIX software (<http://www.genomatix.de/> [160]) for binding sites. Predefined matrices were used (V\$IRF1.01 V\$ISGF3G.01 V\$ISRE.01 V\$ISRE.02V\$CREL.01 V\$NFKAPPAB.01 V\$NFKAPPAB.02 V\$NFKAPPAB65.01 V\$STAT.01 V\$STAT1.01 V\$STAT1.02). Only sites with core similarity above 0.85 were selected. Start indicates position of ATG codon.

To further elucidate the role of STAT1 and IRF1 in tubular cells, we confirmed their expression by performing qPCR (Fig. 2-8). Abundance of STAT1 mRNA was in line with western results for tubular cells (Fig. 2-6 A, B). Treatment with IFNγ resulted in high amplification of STAT1 and IRF1 which was not significantly different after incubation with LPS. Subsequently, we verified NFκB activity in tubular cells. For that reason we analyzed expression of TNFα. Abundance of this cytokine depends directly on the activity of NFκB but not STAT1. Indeed, treatment with LPS resulted in increased expression of TNFα. Prestimulation with IFNγ slightly but not

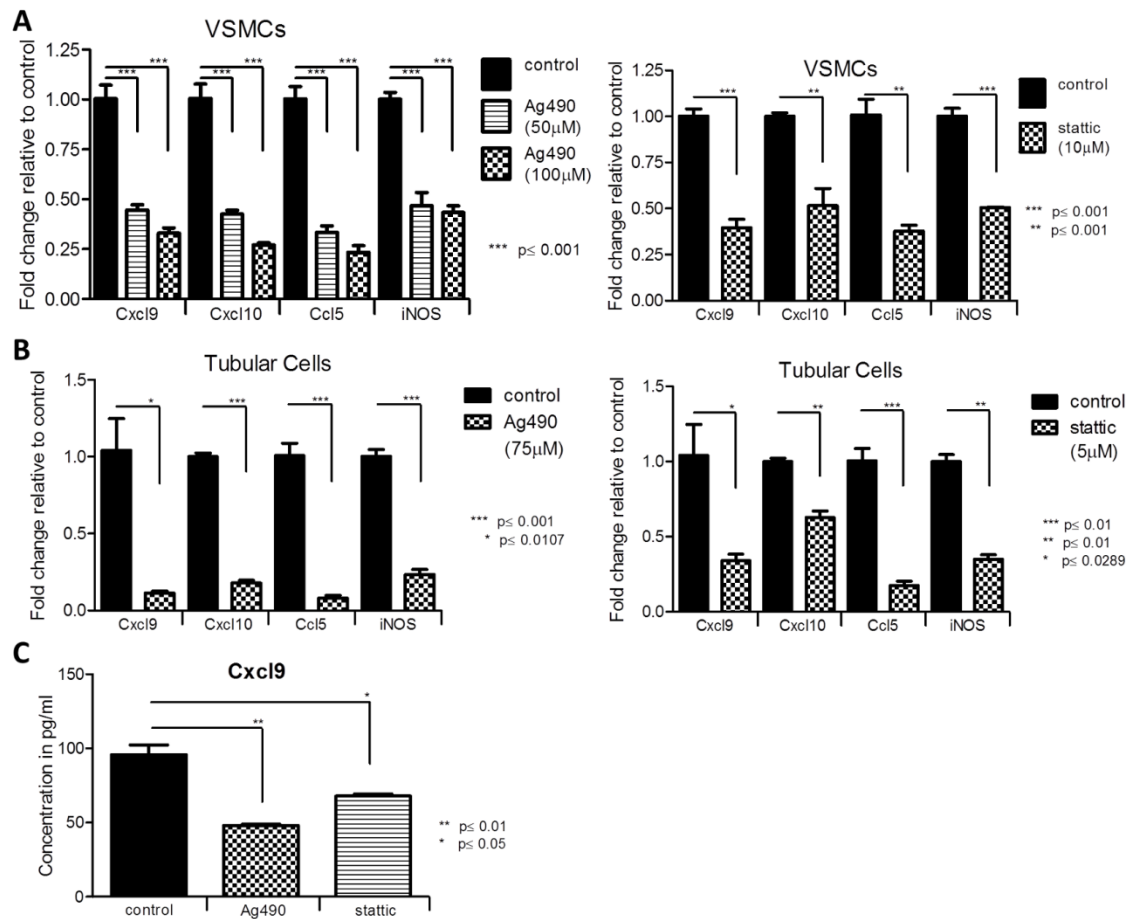
significantly increased levels of  $\text{TNF}\alpha$  suggesting that  $\text{NF}\kappa\text{B}$  activity is not the primary factor that contributes to the synergistic amplification.



**Fig. 2-8. Expression of STAT1, IRF1 and marker of  $\text{NF}\kappa\text{B}$  activity,  $\text{TNF}\alpha$  in tubular cells.** Primary proximal tubular cells were treated with 10ng/ml of  $\text{IFN}\gamma$  for 8h or 1  $\mu\text{g}/\text{ml}$  of LPS for 4h, or both. RNA was isolated and subjected to qRT-PCR for *STAT1*, *IRF1* and *TNF $\alpha$* .  $p < 0.05$  was considered significant. N/S - not significant.

#### **Ag-490 and Stattic attenuates STAT1-dependent crosstalk between $\text{IFN}\gamma$ and LPS.**

To obtain further evidence for a role of STAT1 and JAK/STAT pathway in cross-talk between  $\text{IFN}\gamma$  and LPS, we treated VSMCs and proximal tubular cells with  $\text{IFN}\gamma$  and LPS in the absence or presence of inhibitors that are known to affect either JAK2 (Ag490) or STAT1 (stattic) (Fig. 2-9). Indeed, Ag490 and stattic diminished expression of *Cxcl10*, *Cxcl9*, *Ccl5*, *Nos2* in VSMCs and in proximal tubular cells (Fig. 2-9 A and B) compared to controls treated with  $\text{IFN}\gamma$  and LPS. Attenuated response to  $\text{IFN}\gamma$  and LPS in proximal tubular cells was confirmed by ELISA for *Cxcl9* (Fig. 2-9 C).



**Fig. 2-9. Crosstalk between IFN $\gamma$  and LPS in VSMCs and proximal tubular cells is inhibited in the presence of Ag490 or static.** Cells were treated with 10ng/ml of IFN $\gamma$  for 8h and 1  $\mu$ g/ml of LPS for 4 hrs. **A**, VSMCs were pre-treated with Ag490 or Static for 12h and then treated as above. RNA was isolated and subjected to qRT-PCR for *Cxcl10*, *Cxcl9*, *Ccl5* and *iNOS*. **B**, Proximal tubular cells. B. Proximal tubular cells were pre-treated with Ag490 or Static for 12h and then treated as above. RNA was isolated and subjected to qRT-PCR for *Cxcl9*, *Cxcl10*, *Ccl5* and *iNOS*. **C**, On the medium remained after treatment as in B, ELISA for *Cxcl19* was performed.  $p < 0.05$  was considered significant.

## Discussion

The pleiotropic functions of IFN $\gamma$  and LPS cannot be explained only by separate action of individual transcription factors such as STAT1 or NF $\kappa$ B. Indeed, another mechanism (called priming) by which IFN $\gamma$  and TLR4 ligands achieve strong responsiveness was observed in immune cells. It was shown that prestimulation of macrophages with IFN $\gamma$  and subsequent treatment with TLR4 agonists greatly amplified expression of downstream-dependent genes [152]. This crosstalk between IFN $\gamma$  and TLR4 has a fundamental role in host response against pathogens, but it can also participate in the pathophysiology of many diseases. To date there

is limited information about potential signal integration between IFN $\gamma$  and TLR4 in non-immune cells. Our results suggest that also in ECs, VSMCs and proximal tubular cells, crosstalk between IFN $\gamma$  and LPS results in amplification of genes related to inflammation.

Several mechanisms have been suggested by which the IFN $\gamma$  and TLR4 pathway can cooperate. Experiments performed in macrophages revealed that in addition to IFN $\gamma$  also TLR4 stimulation triggers phosphorylation of STAT1 at tyrosine 701. LPS-mediated phosphorylation of STAT1 is utilized by the induction and subsequent autocrine activities of type I IFN (IFN $\beta$ ) and as such this crosstalk is protein synthesis-dependent. Indeed, we observed STAT1 phosphorylation upon treatment with LPS, which was blocked in the presence of cyclohexamide, the substance that inhibits protein synthesis (data published here [161]). One of the models explaining functional cooperation between IFN $\gamma$  and LPS in macrophages was a mechanism suggested by Schroder et al. [120]. In this model the transactivator ability of STAT1 is highly amplified upon treatment with both IFN $\gamma$  and TLR ligands. Similarly to macrophages and splenocytes (Fig. 2-1), treatment of ECs and VSMCs with IFN $\gamma$  followed by LPS resulted in increased STAT1 phosphorylation as compared to both factors alone. Stimulation with IFN $\gamma$  followed by LPS revealed a synergistic amplification of *Cxcl9*, *Cxcl10*, *Ccl5*, and an important contributor of oxidative stress, *Nos2* (iNOS), in vascular cells as well as in proximal tubular cells. This coincided with increased STAT1 phosphorylation in ECs and VSMCs. Taken together, our results suggest that also in vascular cells increased STAT1 phosphorylation and thus transactivator ability is mediated by TLR-dependent expression of type I IFN.

Interestingly, pretreatment of proximal tubular cells with IFN $\gamma$  followed by LPS did not result in amplified phosphorylation of STAT1 (Fig. 2-4). Wen et al. suggested the importance of serine 727 phosphorylation in maximal STAT1 activity [54]. Thus we verified whether this phenomenon occurs in proximal tubular cells. We observed increased serine phosphorylation of STAT1 upon treatment with IFN $\gamma$  that was not changed upon addition of LPS. Together with the lack of response to LPS, these results suggest the existence of other, phosphorylation-independent and tissue-specific mechanisms that are involved in functional cooperation between IFN $\gamma$  and TLR4 in proximal tubular cells. One mechanism which may explain this amplification may be related to increased expression of STAT1 and other transcription factors



that are STAT1-dependent, e.g. IRF1. Indeed, promoter analysis predicted the presence of binding sites for NFκB and IRF1 in the regulatory regions of *Cxcl9*, *Cxcl10*, *Ccl5* and *Nos2* (Fig. 2-7). Expression analysis of IRF1 confirmed a higher abundance of IRF1 and STAT1 upon treatment with IFNγ that was not affected by LPS (Fig. 2-8). Because phosphorylation of STAT1 does not seem to play a role, either increased interaction of STAT1 or IRF1 or activity of NFκB may contribute to synergistic amplification. We examined the expression of TNFα to verify whether the transcriptional activity of NFκB is increased during stimulation with both stimuli. Literature data indicate that abundance of this cytokine depends directly on the activity of NFκB but not STAT1 [143]. We observed induction of TNFα expression upon LPS stimulation that was not significantly different from the expression observed in samples treated with IFNγ and LPS. These data suggest that not the transcriptional activity of NFκB but increased expression of IRF1 and STAT1 upon IFNγ stimulation contribute to the synergistic amplification of proinflammatory mediators in tubular cells. A higher abundance of STAT1 and IRF1 may lead to increased sensitivity to LPS and, as a consequence, to amplification of *Cxcl10*, *Ccl5* and *Nos2*.

Recently, a new model which may also explain the mechanism of signal integration between IFNγ and TLR4 was proposed [85]. In this model STAT1 is considered to be a factor that initiates chromatin remodeling. IFNγ-activated STAT1 affects histone acetylation and thus causes increased and prolonged recruitment of additional transcription factors and polymerase II after TLR4 stimulation. Further studies are necessary to clarify the mechanism contributing to signal integration in proximal tubular cells.

The transcriptional regulation of proinflammatory mediators has shown to involve several transcription factors, including STAT1, NFκB or IRFs [129, 136, 137, 162-166]. Indeed, *in silico* promoter analysis predicted the presence of binding sites recognized not only by STAT1 but also other factors such as NFκB or IRF1 (Fig. 2-7). Our experiments suggest that cooperation between STAT1, IRF1 and other transcription factors is crucial for synergistic amplification of *Cxcl9*, *Cxcl10*, *Ccl5* and *Nos2* also in vascular cells as well as in tubular cells. Expression of *Nos2* was highly amplified upon combined treatment in all investigated cells. Additionally, this result was confirmed by measurements of nitrite in VSMCs (Fig. 2-3 C). The promoter of the *Nos2* gene contains regulatory sequences recognized not only by NFκB but also by STAT1 homodimer complexes, (gamma interferon-activated site, GAS) and IRFs [167-170] (Fig. 2-7).

In addition, recently published data pointed to the important role of the ISGF3 complex (containing STAT1-STAT2-IRF9) involved in the regulation of *iNOS* expression upon concomitant stimulation with type I IFN and the agonist of pathogen recognition receptors [164]. In macrophages, combined stimulation with IFN $\gamma$  and LPS or TNF $\alpha$  results in increased upregulation of NO [171]. Foremost, this upregulation is IRF1-dependent, thus suggesting cooperation between STAT1, IRF1 and NF $\kappa$ B in response to IFN $\gamma$  and LPS [169]. Taking into consideration the fact that the abundance of *Nos2* after stimulation with IFN $\gamma$  was barely detectable, it is tempting to speculate that transcription of *iNOS* is rather dependent on the ISGF3 complex than on functional cooperation between STAT1 homodimers and IRF1.

Similarly to *iNOS*, expression of *Cxcl10* was synergistically increased in ECs and VSMCs treated with IFN $\gamma$  followed by LPS, and was ameliorated in the presence of Ag490 or stattic. This result correlates with a predominant STAT1-dependent mechanism engaged in the integration of both stimuli. Indeed, literature data suggest that, like *iNOS*, maximal expression of *Cxcl10* requires activation of both pathways. However, in contrast to *Nos2* transcription, synergistic amplification of *Cxcl10* requires cooperation between STAT1 and IRF1, but not NF $\kappa$ B [136, 163, 172]. Importantly, experiments performed in our laboratory revealed that also in the vascular cells, transcription of *Cxcl10* is protein synthesis-dependent [161]. Because expression of IRF1 can be triggered not only by IFN $\gamma$  but also by LPS, it is very likely that a similar IRF1-dependent mechanism mediates expression of *Cxcl10* in VSMCs and ECs upon treatment with IFN $\gamma$  and LPS. However, in tubular cells, LPS stimulation did not result in a statistically significant increase in IRF1 expression. Thus it is very likely that NF $\kappa$ B plays a more substantial role in regulating *Cxcl10* abundance in tubular cells.

In contrast to *iNOS* and *Cxcl10*, whose expression pattern was similar for all investigated cell types, abundance of *Cxcl9* and *Ccl5* was different between cells isolated from spleens and non-immune cells. While there was strong transcriptional activation of *Ccl5* and *Cxcl9* upon combined treatment in vascular cells and tubular cells, we could not detect a synergistic effect of IFN $\gamma$  and LPS in murine splenocytes (Fig. 2-1). This phenomenon can be explained by the partially different transcriptional regulation of specified genes in myeloid and lymphoid cells. Literature data suggest that in immune cells, expression of *Cxcl9* and *Ccl5* is controlled by tissue-specific transcription factors [144, 173]. These factors are often present in the latent

stage, therefore additional stimulation with extracellular ligands (e.g. IFN $\gamma$  or LPS) is not mandatory for efficient upregulation of downstream targets; for example, IFN $\gamma$ -mediated transcription of Cxcl9 is dependent on cooperation between STAT1 and tissue-specific transcription factor Pu.1 in myeloid cells [173].

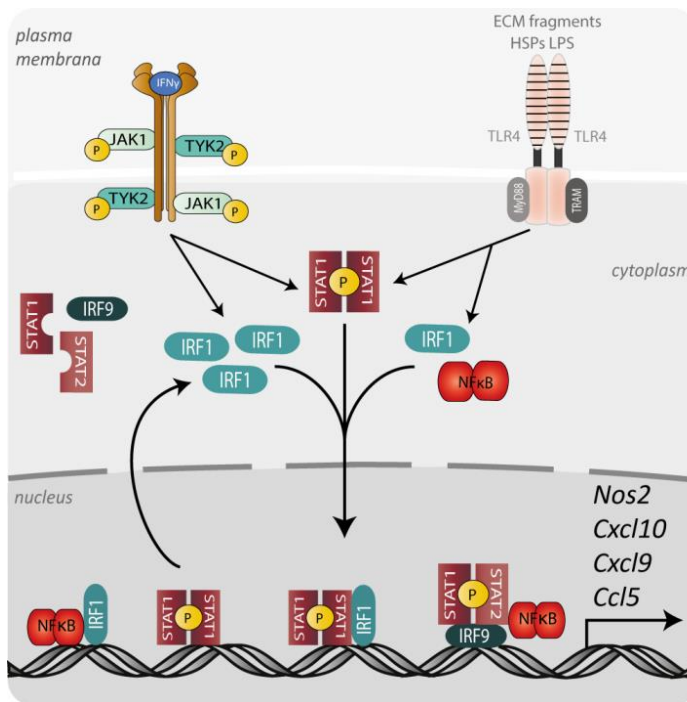
Although the patterns of expression upon combined treatment are similar for Cxcl9 and Ccl5, treatment with LPS or IFN $\gamma$  alone indicates different regulatory mechanisms. As opposed to Cxcl9, whose expression is IFN $\gamma$ -dependent, transcription of Ccl5 relies rather on activation of TLR4. Despite the fact that IFN $\gamma$  stimulation leads to expression of Ccl5 in macrophages [174], in other cell types, e.g. synovial fibroblasts, mesothelial cells, alveolar epithelial cells or peritoneal fibroblasts, stimulation only by IFN $\gamma$  is insufficient for expression of Ccl5 [175-179]. This is in line with our observations for vascular and tubular cells. Indeed, literature data suggest the importance of NF $\kappa$ B in the regulation of Ccl5 expression [137, 178].

To obtain further evidence for the role of the STAT1 and JAK/STAT pathway in crosstalk between IFN $\gamma$  and LPS, we used inhibitors that are known to affect either JAK2 (Ag490) or STAT1/STAT3 (stattic) [180]. Expression of STAT1-dependent genes was only partially attenuated upon stimulation with the antagonist of the JAK/STAT pathway (Fig. 2-7). Ag490 is an inhibitor of the JAK2 kinase which participates in the formation of active STAT1 dimers upon stimulation with IFN $\gamma$ . Indeed, Ag490 can effectively block the IFN $\gamma$  response, as samples treated only with IFN $\gamma$  did not express STAT1-dependent genes (data not shown). However, during the crosstalk, STAT1 phosphorylation is partially mediated through the activity of JAK1 and Tyk2 kinases. This mechanism could explain only the partial inhibition of STAT1 action by Ag490 and further indicates the importance of the autocrine activities of type I IFN (IFN $\beta$ ).

Although stattic was considered as a specific inhibitor of STAT3, experiments performed in our laboratory revealed that it can also antagonize STAT1 phosphorylation upon stimulation with type I interferon [180, 181]. However, experiments based on IFN $\gamma$  stimulation, which is a far more powerful activator of STAT1, revealed only partial efficacy of stattic. This suggests that STAT3 is indeed a primary target of stattic and that partial inhibition of STAT1 action is rather a side effect of stattic, as both STATs share structural similarities. Importantly, both inhibitors can affect STAT1 and STAT3 action. Thus, we cannot exclude the role of STAT3 in upregulation

of these genes. Further experiments with STAT1- and STAT3-deficient animal models are mandatory.

Our results presented in this section as well as those published in the *American Journal of Physiology – Cell Physiology* [161] provide further evidence for the crosstalk between IFN $\gamma$  and TLR in ECs, VSMCs and proximal tubular cells. Although the mechanisms of transcription of genes prone to synergistic amplification may vary in detail, one common feature is the involvement of STAT1 and IRF1 in the regulation of amplified genes.



**Fig. 2-10. STAT1 as a central point of crosstalk between IFN $\gamma$  and TLR4 induced pathways.** Treatment with IFN $\gamma$  leads to increased expression of STAT1 and STAT1-dependent transcription factor that participate in the TLR4 signaling - IRF1. Increased STAT1-dependent expression of the IRF1 and their subsequent collaboration with other transcription factors resulted in synergistic amplification of *Nos2*, *Cxcl10*, *Cxcl9* and *Ccl5*.

Similarly to splenocytes, stimulation with IFN $\gamma$  and TLR4 in ECs and VSMCs resulted in augmented STAT1 phosphorylation and increased expression of the chemokines *Cxcl9*, *Cxcl10*, *Ccl5* and *Nos2*. Inhibition of JAK2 (Ag490) or STAT1 phosphorylation (stättic) partially prevented this effect. In proximal tubular cells it was not augmented STAT1 phosphorylation but rather increased abundance of IRF1 that contributed to the synergistic amplification.

Altogether, STAT1 and IRF1 could potentially represent a novel target of therapeutic intervention that would have a crucial role in mediating the interplay between damaged organ and host immunity in order to control progression of inflammation mediated by IFN $\gamma$  and TLR4.

## **Chapter 3**

### **STAT1 and IRF8 orchestrate IFN $\gamma$ and LPS-mediated signal integration in the vasculature that leads to amplified pro-atherogenic responses**

#### **Introduction**

A variety of diseases, including those which affect the cardiovascular system, have pathophysiological important role of the immune component. Atherosclerosis is a type of arteriosclerosis in which the function of the artery is affected by the accumulation of fatty plaques and cholesterol in the vessel wall. Recent studies have provided evidence for the crucial role of inflammation in all stages of the disease, starting from early endothelial cell (EC) dysfunction and altered contractility of vascular smooth muscle cells (VSMCs) through recruitment of blood leukocytes to the injured vascular wall and, ultimately, thrombus formation in the lumen [11, 182, 183]. Interferon (IFN) $\gamma$  is a pivotal mediator of innate and adaptive immunity. Since the discovery that IFN $\gamma$  is highly expressed in lesions, its role in atherosclerosis has been broadly studied [63, 68]. IFN $\gamma$  mediates its own action through activation of the JAK/STAT pathway. Binding of this cytokine to IFNGR receptors leads through phosphorylation and homodimer formation to transcriptional activation of the protein called the signal transducer and activator of transcription (STAT) 1. Recently obtained data indicate that STAT1 is not only involved in signal transduction upon treatment with IFN $\gamma$  but also contributes to the biological response to different toll-like receptors (TLRs). TLRs are a family of innate immune pattern-recognition receptors which recognize pathogen- and damage-associated molecular patterns, e.g. lipopolysaccharide (LPS), or heat shock proteins (HSP), and play an important role in the progression of atherosclerosis [184]. Activation of TLR4 signaling triggers the induction of various target genes that include those encoding type I IFNs, chemokines and cell surface molecules [150]. Some of these genes are regulated secondary to LPS-induced IFN $\beta$  which, after secretion, binds to the type I IFN receptor to activate gene expression in a STAT1-dependent manner [185]. As such, STAT1 has been identified as an important mediator in the biological response to different TLRs, including TLR4. Signal integration between IFN $\gamma$  and TLRs has been described especially in immune cells

and was related to host defense against pathogens and injury. Stimulation of macrophages and dendritic cells with IFN $\gamma$  and LPS was mandatory for efficient expression of proinflammatory mediators [120, 121]. Moreover, STAT1 was identified as an important mediator of this crosstalk [118, 128]. In addition to STAT1, also STAT1 target genes which belong to the family of interferon regulatory factors (IRF) have been involved in signal integration between IFN $\gamma$  and LPS; for instance, IRF8, which was thought to be immune specific, was identified as being involved in the synergistic induction of proinflammatory genes, such as IL1, IL6, IL12, TNF $\alpha$  and Ccl5. [115, 144]. These data imply that STAT1 and the IRFs coordinate the crosstalk between IFN $\gamma$  and TLRs and therefore positively regulate inflammation. Our recent observations suggest that the mechanism that was previously identified in immune cells is also present in cells that build the vascular wall [161]. Augmented STAT1 phosphorylation was associated with increased expression of chemokine CXCL10 and the adhesion molecule ICAM-1. We could observe increased adhesion of U937 leukemia cells to ECs in a STAT1-dependent manner [161].

In this chapter we provide results to further support the hypothesis that activated STAT1 together with downstream-regulated IRFs serve as a platform for increased TLR4 signaling in cells from the vasculature, thus resulting in the expression of genes related to inflammatory processes. We conducted expression profiling on VMSCs in order to identify sets of STAT1 target genes prone to synergistic amplification. We identified sets of new, potentially interesting targets. We showed for the first time that transcription factor IRF8 is also expressed in the vessel wall and may be involved in the progression of inflammatory response. Moreover, by performing immunohistochemistry on human data sets we provided further evidence for the importance of the above-mentioned signaling in human atherosclerosis.

## **Material and Methods**

### **Cell culture experiments**

WT mice (strain background *C57BL/6*) were obtained from Charles River Laboratories (Sulzfeld, Germany). *STAT1*<sup>-/-</sup> and *IRF8*<sup>-/-</sup> mice (both *C57BL/6* background) were kindly provided by Thomas Decker and Carol Stocking, respectively [186], *TLR4*<sup>-/-</sup> (*C57BL/6* background) were

bred in our own facility [187]. Primary murine Vascular Smooth Muscle cells (VSMCs) were isolated from *WT* or *STAT1*<sup>-/-</sup> or *IRF8*<sup>-/-</sup> mice by enzymatic digestion [188] in a solution containing collagenase type II 1mg/ml, soybean trypsin inhibitor 1mg/ml (Life Technologies, Carlsbad, USA), elastase 0.744u/ml (Sigma-Aldrich, Missouri, USA) in HBSS (Life Technologies). Isolated aortas from 2 mice were cleaned from perivascular fat and predigested for 10min. Subsequently adventitia was removed, aortas were cut lengthwise and intima was removed by gentle scraping. Next aortas were enzymatically digested for 1h at 37°C. After digestion aortas were passed through 100µm cell strainer (BD, Heidelberg, Germany) and left undisturbed on 3 wells of a 48 well plate for 1 week. Until passage number 3 cells were cultivated in DMEM medium (PAA, Linz, Austria) containing 4.5mg/l Glucose, 2mM L-Glu, supplemented with 100U/ml penicillin and 100µg/ml streptomycin and 20% FBS (PAA). After 3<sup>rd</sup> passage 10% FBS was used. Human Microvascular Endothelial Cells (HMECs) obtained from Centers for disease control and prevention (Atlanta, GA, USA) were cultivated in MCDB-131 (Life Technologies) medium containing 10% FBS (PAA), 100U/ml penicillin, 100µg/ml streptomycin, 0.01µg/ml EGF, 0.05µM hydrocortisone, 2mM L-glutamine (PAA). On the day before an experiment for both cell types full medium was exchanged into medium containing 2% serum. Afterwards cells were treated with 10ng/ml of IFN $\gamma$  (Life Technologies) and/or 1µg/ml of LPS (Sigma).

### **RNA isolation and real-time PCR**

Total RNA was isolated from VSMCs and HMECs using RNAeasy Mini Kit (Qiagen, Hilden, Germany) together with DNase digestion step according to the manufacture's protocol. Complementary DNA was synthesized using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, USA), according to manufacturer's protocol. Quantitative reverse transcriptase PCR (qRT-PCR) was performed using SSoFast Evagreen (MyiQ ICycler, Bio-Rad). Forward and reverse primers are depicted in Table 3-1. The 2<sup>-ddCt</sup> method was applied for quantification [155]. Fold change in the target gene were normalized to *GAPDH* and relative to the mean expression at untreated sample. The results are expressed as fold of control from at least 3 independent assays. Regular PCR was performed using AmpliTaq Gold 360 DNA polymerase kit (Life Technologies) together with dNTP mix (Sigma). Bands were visualized by staining gels with peqGreen (Peqlab).

**Table 3- 1. Primer sequences used in experimental procedures**

Gene Name	Forward	Reverse
<b>Gapdh</b>	TCGGTGTGAACGGATTTGGC	TTTGGCTCCACCCTTCAAGTG
<b>Irf8</b>	GCAGGATGTGTGACCGGAAC	CCACCTCCTGATTGTAATCCT
<b>Ccl5 (Rantes)</b>	CGCACCTGCCTCACCATAT	CACTTCTTCTCTGGGTGGC
<b>Cxcl10</b>	TCATCCCTGCGAGCCTATCC	GGAGCCCTTTTAGACCTTTT
<b>Cxcl9</b>	CTGCCATGAAGTCCGCTGTTCT	TCCCCCTCTTTTGCTTTTCTT
<b>Ccl12</b>	AGCTACCACCATCAGTCCTCA	CAAGGATGAAGGTTTGAGACG
<b>Ccr12</b>	ACAGTACGACCTCCACAAGC	GGAACAGGCTGCGAAGGTAT
<b>β-actin</b>	CCAGCCTTCCTTCTGGGTAT	ACTCATCGTACTCCTGCTTGC
<b>GAPDH_human</b>	CAACTGCTTAGCACCCCTGG	CAGGTCAGGTCCACCACTGA
<b>IRF8_human</b>	GGGAGAATGAGGAGAAGAGCA	CCGCACTCCATCTCTGTAAT

### Microarray analysis

VSMCs from WT and STAT1<sup>-/-</sup> were treated as described in Fig. 1. RNA from control and treated samples was isolated and labeled according to Illumina® TotalPrep™ RNA Amplification Kit (LifeTechnologies, CA). Standard Illumina Expression BeadChip MouseRef-8v2 (Illumina, SA) hybridization protocol was used to obtain the raw data. Chips were scanned using HiScanSQ system. The complete data of the Illumina Expression BeadChip analysis can be found at the NCBI GEO, with the accession number GSE49519. The average gene expression signals from 3 independent biological experiments were taken for statistical testing. Only genes from treated samples with detection p-value <0.05 were chosen for further analysis. Background subtraction and quantile normalization were used to obtain statistically significant (p<0.05) at least 2-fold upregulated genes. Genes which expression after co-treatment was at least 2-fold higher upon stimulation with IFNγ + LPS as compared to the sum of the treatments with both factors alone were considered as amplified. Promoters for amplified STAT1 dependent genes were screened using GENOMATIX software (<http://www.genomatix.de/>) [160]. The promoter regions from -1000 to +100bp were searched for binding sites (V\$IRF1.01 V\$ISGF3G.01 V\$ISRE.01 V\$ISRE.02 V\$CREL.01 V\$NFKAPPAB.01 V\$NFKAPPAB.02 V\$NFKAPPAB65.01 V\$STAT.01 V\$STAT1.01 V\$STAT1.02) or models with core similarity at least 0.85.



### **Western blot analysis**

Total IRF8, STAT1 (Santa Cruz, sc6058, sc346), GAPDH and phosphorylated STAT1 (Cell Signaling, 5174s, 9171l) were determined by western blotting in VSMCs and HMECs. After treatment cells were homogenized in a Ripa lysis buffer (Sigma-Aldrich) containing phosphatases and proteases inhibitors (Roche). Protein concentration was determined using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific). 40µg of protein per lane was loaded and resolved by SDS-poly-acrylamide gel electrophoresis (PAGE) under reducing conditions. Proteins were transferred onto PVDV (Millipore, Billerica, USA) membrane. After incubation with primary and horseradish peroxidase-conjugated secondary antibodies (Santa Cruz), immunoreactivity was detected by adding Luminata Forte Western Substrate (EMD Millipore) and measured by INTAS imaging system (Intas, Goettingen, Germany).

### **Cytokine detection ELISA**

Expression of murine Cxcl10, Ccl5 (Peprotech, Hamburg, Germany) as well as Cxcl9 (Sigma) was performed on medium remained after treatment of VSMCs using sandwich ELISA tests according to the manufacturer's instructions.

### **Measurement of nitric oxide (NO)**

VSMCs were treated as depicted in cell experiment section. After treatment medium was refreshed and cells were cultivated for further 24h. Subsequently medium was collected and 100ul was used to measure amount of NO by Griess diazotization reaction. Medium was incubated with freshly prepared solution containing 1% sulfanilamide 5% HCl, 0.1% aqueous solution of 2-(1-Naphthylamino)ethylamine dihydrochloride (Sigma). After 10min incubation OD at 560nm was measured and compared to the standard curve.

### **Histology and immunohistochemistry**

Histological analyses and immunohistochemistry were performed on representative sections (2-3 µm) of formalin fixed in paraffin embedded tissue samples from six human carotid atherosclerotic lesions and four healthy controls. Hematoxylin-Eosin (HE) and Elastica-van-Gieson (EvG) staining were performed in order to assess sample morphology. For characterisation of the cells within atherosclerotic plaques, specimens were treated with

antibodies against vascular smooth muscle cells (smooth muscle myosin heavy chain 1 and 2 (SM-M10), rabbit polyclonal, dilution 1:4.000 (Abcam, ab81031) and endothelial cells (anti-CD31, mouse monoclonal, clone JC70A, dilution 1:100; Dako).

For the detection of specific cytokines, CXCL9 (MIG) and CXCL10 (IP10), as well as the phosphorylated transcription factor STAT1, following primary antibodies were used: rabbit polyclonal anti-MIG (Abcam, ab9720; dilution 1:500), rabbit polyclonal anti-IP10 (Abcam, ab47045; dilution 1:200), and rabbit monoclonal phospho-Stat1 (Cell Signaling, 91711; dilution 1:400). All antibodies were first optimised on tonsil using different dilutions, staining conditions and with or without blocking. Optimal results were achieved by blocking anti-MIG and anti-phospho-Stat1 with goat serum, anti-IP10 without the blocking procedure.

Following incubation with primary antibody visualisation was performed by peroxidase/DAB ChemMate Detection Kit according to the manufacturer's instruction (biotinylated goat anti-mouse/anti-rabbit secondary antibody; Dako).

### **Histology and fluorescent immunohistochemistry**

Histological analyses and fluorescent immunohistochemistry were performed on representative sections (2-3  $\mu\text{m}$ ) of formalin fixed, paraffin embedded human carotid artery tissue specimens obtained from patients with high-grade carotid artery stenosis (>70%) after carotid endarterectomy in the Department of Vascular and Endovascular Surgery (Klinikum rechts der Isar, Technical University Munich). Immunostaining was performed using antibodies to detect VSMCs (mouse monoclonal anti-SMA, HHF35, dilution 1:200; Dako, Glostrup, Denmark), endothelial cells (mouse monoclonal anti-CD31, JC70A, dilution 1:100; Dako), mouse monoclonal macrophages/monocytes (anti-CD68, KP1, dilution 1:1000; Dako), leukocytes (rabbit polyclonal anti-CD45, dilution 1:200; Dako) and IRF8 (goat polyclonal antibody, dilution 1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Primary antibodies (combined in the following way: CD68/IRF8, CD45/IRF8, CD31/IRF8, SMA/IRF8) were incubated over night at 4°C, and visualized by secondary antibody incubation for one hour (Alexa Fluor 488-conjugated goat-anti-mouse IgG, Alexa Fluor 488-conjugated donkey-anti-rabbit, or Cy3-conjugated donkey-anti-goat, dilution 1:200; Dianova, Hamburg, Germany). VECTASHIELD mounting medium containing DAPI fluorescence dye for staining of the cell

nuclei was used (Axxora, Loerrach, Germany). Images were recorded using a Leica microscope DM4000B (Leica Microsystems, Wetzlar, Germany).

### **Migration assay**

Migration assay was performed according to Guo et al [189]. Briefly,  $10^6$  of isolated red blood cells depleted splenocytes were loaded into the upper chamber of Transwell 24-well plates (Corning). The bottom chamber was filled with 600ul of the medium collected after treatment of VSMCs with LPS, IFN $\gamma$  or IFN $\gamma$  and LPS. After incubation for 3h at 37°C, migrated cells were stained with CD45FITC and CD3APC antibody (Miltenyi Biotec 130091609, 130092977) and analyzed by flow cytometer (Miltenyi Biotec).

### **Ex vivo experiments and contractility studies**

Cleaned form perivascular fat aortas were cut into 2mm long rings (for myograph) and placed in DMEM medium containing 2% FBS (Sigma). Next, aortas were treated with 10ng/ml of IFN $\gamma$  and/or 1 $\mu$ g/ml of LPS. Vascular contractility studies were performed according to the technique described by Mulvany et al. [190]. After treatment, 2mm long rings were mounted in a 4-channel myograph (620M, Danish Myo Technology, Denmark) in the organ chamber filled with physiological saline solution (PSS; 118.99mM NaCl, 4.69mM KCl, 1.17mM MgSO $_4$ \*7H $_2$ O, 1.18mM KH $_2$ PO $_4$ , 2.5mM CaCl $_2$ \*2H $_2$ O, 25mM NaHCO $_3$ , 0.03mM EDTA, 5.5mM Glucose). During the experiment PSS buffer was aerated with carbogen (95% O $_2$ + 5%CO $_2$ ). After calibration, vessels were pre-stretched to obtain optimal passive tension. Next, vascular functions were analyzed. Contractility was evaluated by substitution of PSS buffer for high potassium physiological saline solution (KPSS; 74.7mM NaCl, 60mM KCl, 1.17mM MgSO $_4$ \*7H $_2$ O, 1.18mM KH $_2$ PO $_4$ , 1.6mM CaCl $_2$ , 14.9mM NaHCO $_3$ , 0.026mM EDTA, 5.5mM Glucose). For testing viability, vessels were subjected to noradrenaline-induced constriction followed by acetylcholine-induced dilation (Sigma). After washing out with PSS buffer and resting for 15 minutes, noradrenaline dose-response curves were performed. Noradrenaline was used in stepwise increased, cumulative concentration ranging from  $10^{-11}$  to  $10^{-6}$  mol/L. To study vasodilatation, sodium nitroprusside (Sigma) was used in concentrations from  $10^{-10}$  to  $10^{-5}$  mol/L.

## Statistical Analysis

Data are presented as mean  $\pm$  SEM of at least 3 experiments  $\pm$ SEM. For comparisons between more than two groups one-way ANOVA with Tukey post-hoc test was used. In all other experiments comparing two groups, Student's t-test was used. A probability value (p) <0.05 was considered statistically significant (GraphPad Prism<sup>®</sup> 5.0). In contractility studies, two-way ANOVA test with Bonferroni post hoc test was used.

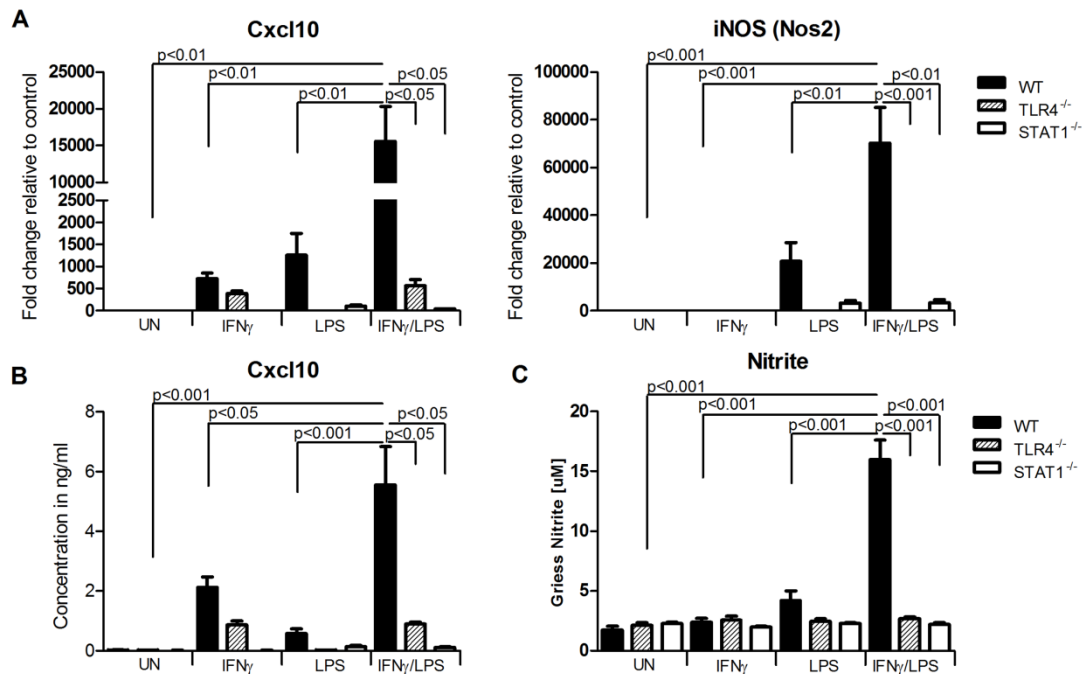
## Results

### In SMCs signal integration between IFN $\gamma$ and LPS is TLR4 and STAT1-dependent.

Our observations presented in previous chapter suggest that both in HMECs and VSMCs, STAT1 orchestrates a platform for cross-talk between IFN $\gamma$  and TLR4. This resulted in augmented STAT1 phosphorylation and increased expression of the genes like chemokine *Cxcl10* and *iNOS* (*Nos2*). In addition, treatment with fludarabine which thought to be a specific STAT1 inhibitor, resulted in ameliorated expression of *Cxcl10* [161, 191, 192]. However, recently obtained data in our laboratory indicate that fludarabine is not a STAT1 specific inhibitor and does affect other STATs [180]. Thus, to confirm that this mechanism is STAT1 dependent, VSMCs were isolated from *WT*, *STAT1*<sup>-/-</sup> and *TLR4*<sup>-/-</sup> mice and treated as depicted in Fig. 3-1. Indeed, we could observe synergistic mRNA amplification of *Cxcl10* (Fig. 3-1 A, left panel) and *iNOS* (Fig. 3-1 A, right panel) in *WT*-VSMCs upon combined treatment, compared with IFN $\gamma$  or LPS alone. Synergistic amplification in *WT*-VSMCs was present at the mRNA level as well as at the protein level for *Cxcl10* (Fig. 3-1 B, left panel). Griess reaction which measures indirectly activity of the nitric oxide synthase, reflected results obtained at mRNA level for *iNOS* in *WT*-VSMCs (Fig. 3-1C). In contrast, this IFN $\gamma$  and LPS-induced signal integration in *Cxcl10* and *iNOS* gene expression was dramatically abrogated in *STAT1*<sup>-/-</sup> and *TLR4*<sup>-/-</sup>-VSMCs (Fig. 3-1 A), which coincided with *Cxcl10* protein levels and amount of the nitrite in the medium (Fig. 3-1B, 3-1 C).

These results further confirm importance of STAT1 in crosstalk between both pathways. Interestingly, whereas TLR4 deficient VSMCs failed to express *Cxcl10* and *iNOS* upon stimulation with LPS, *STAT1*<sup>-/-</sup>-VSMCs not only did not express both genes upon treatment

with IFN $\gamma$  but also treatment with LPS alone resulted with blunted response (Fig. 3-1 A and B). Presented results allow us to suggest that STAT1 is not only involved in the IFN $\gamma$  signal transduction and crosstalk between JAK/STAT and TLR4 but also participates in response to LPS. This could explain lower potency of the response to LPS in *STAT1*<sup>-/-</sup>-VSMCs as compared to *WT*-VSMCs.



**Fig. 3-1. Cxcl10 and iNOS amplification by IFN $\gamma$  and LPS is STAT1 dependent.** **A**, VSMCs were treated with 10ng/ml IFN $\gamma$  for 8h or with 1ug/ml of LPS for 4h or with IFN $\gamma$  for 4h followed by IFN $\gamma$  and LPS for additional 4h. RNA was isolated and qRT-PCR for *Cxcl10* and *iNOS* using *Gapdh* as internal control was performed. **B**, Cells were treated as in A. On the medium remained after treatment ELISA for *Cxcl10* was performed. **C**, After treatment medium was refreshed and cells were cultivated for further 24h. 100 $\mu$ l was used to measure nitrite. Data represent means of at least 3 independent biological experiments  $\pm$ SEM and  $p < 0.05$  was considered as significant.

**Table 3-2. Genes prone to synergistic amplification upon stimulation with IFN $\gamma$  and/or LPS and their promoter analysis**

The Table introduces genes that expression is at least 2-fold higher upon stimulation with IFN $\gamma$ +LPS as compared to the sum of the treatments with both factors alone (see column "Signal integration"). Other numbers represent fold changes compared to control. Cross indicates presence of specific sequence in the promoter regions.

SYMBOL	WT IFN $\gamma$	WT LPS	Signal integration	WT IFN $\gamma$ +LPS	STAT1 <sup>-/-</sup> IFN $\gamma$	STAT1 <sup>-/-</sup> LPS	STAT1 <sup>-/-</sup> IFN $\gamma$ +LPS	STAT_NFkB	IRF_NFkB	ISRE	STAT	NFkB	Cluster
<b>Cxcl9</b>	150.73	20.25	15.46	2643.50	0.93	9.87	9.52	x	-	-	-	-	A
<b>Cxcl10</b>	343.62	665.68	2.25	2273.44	0.47	119.63	66.53	x	x	-	-	-	
<b>Rsad2</b>	22.91	209.45	2.19	509.05	1.53	2.93	2.71	-	-	x	x	x	
<b>Gbp5</b>	53.48	82.66	2.85	388.28	1.28	65.64	72.68	x	-	x	x	x	
<b>Batf2</b>	134.37	3.23	2.29	314.46	5.35	5.10	8.31	x	-	x	x	x	B
<b>Ubd</b>	21.54	3.38	13.11	326.57	0.80	4.71	6.44	x	-	x	-	-	
<b>Cd74</b>	37.66	2.93	5.09	206.50	0.78	8.01	8.24	x	x	-	-	-	
<b>Fam26f</b>	43.02	13.60	2.42	137.16	0.95	13.25	13.07	-	-	x	x	-	
<b>Serpina3g</b>	24.76	13.36	2.59	98.56	2.12	5.12	7.79	x	-	x	-	-	
<b>Ccl5</b>	2.14	249.59	2.04	512.54	0.83	64.48	52.22	x	x	-	-	-	C
<b>Tnfaip2</b>	6.52	45.55	2.27	118.00	1.14	28.83	27.04	x	-	-	-	-	
<b>Cd40</b>	1.03	15.21	7.83	127.14	0.70	14.09	14.14	-	-	x	x	x	
<b>LincR</b>	1.07	21.38	4.84	108.75	1.16	29.33	25.78	-	-	x	x	x	
<b>Nos2</b>	0.71	33.99	2.08	72.15	0.73	22.52	24.13	x	x	-	-	-	
<b>Ccl2</b>	4.18	8.16	3.72	45.93	0.76	5.87	4.65	x	-	x	-	-	D
<b>Mx1</b>	3.10	12.73	2.49	39.41	0.91	1.68	1.18	x	-	x	-	-	
<b>Has1</b>	1.40	8.71	2.16	21.84	1.17	11.64	14.92	-	-	-	x	x	
<b>Oasl1</b>	1.80	7.61	2.13	20.10	1.03	1.45	1.83	-	-	x	-	x	
<b>MCP-2</b>	6.12	3.75	3.83	37.77	5.21	44.53	58.31	-	-	-	x	-	
<b>Atf3</b>	3.73	2.47	3.27	20.29	1.21	4.34	3.96	-	-	-	x	x	
<b>Ifi205</b>	5.39	2.99	2.28	19.10	0.64	4.24	4.81	-	-	x	x	-	
<b>Upp1</b>	2.67	2.37	2.87	14.45	1.17	2.25	3.53	x	-	x	-	-	
<b>Tnfrsf11a</b>	0.20	5.87	2.12	12.85	1.46	7.44	12.33	x	-	-	-	-	
<b>Irf8</b>	6.13	0.41	2.98	19.51	1.40	1.03	0.77	x	-	-	-	-	E
<b>MCP-5</b>	1.05	0.32	8.65	11.86	0.69	0.59	0.78	-	-	x	x	x	
<b>Sectm1a</b>	1.21	0.27	2.45	3.62	0.30	0.37	1.16	x	-	x	-	-	
<b>Gja4</b>	1.25	0.83	4.50	9.35	0.55	0.37	0.27	-	-	-	-	x	
<b>Egr2</b>	1.74	1.96	1.98	7.32	0.98	2.31	1.73	x	-	-	-	-	
<b>Itpk1</b>	1.12	1.29	2.19	5.29	0.86	0.97	1.30	-	-	-	x	x	
<b>Etsrp71</b>	0.48	0.83	2.43	3.18	1.39	0.96	0.96	-	-	-	x	x	

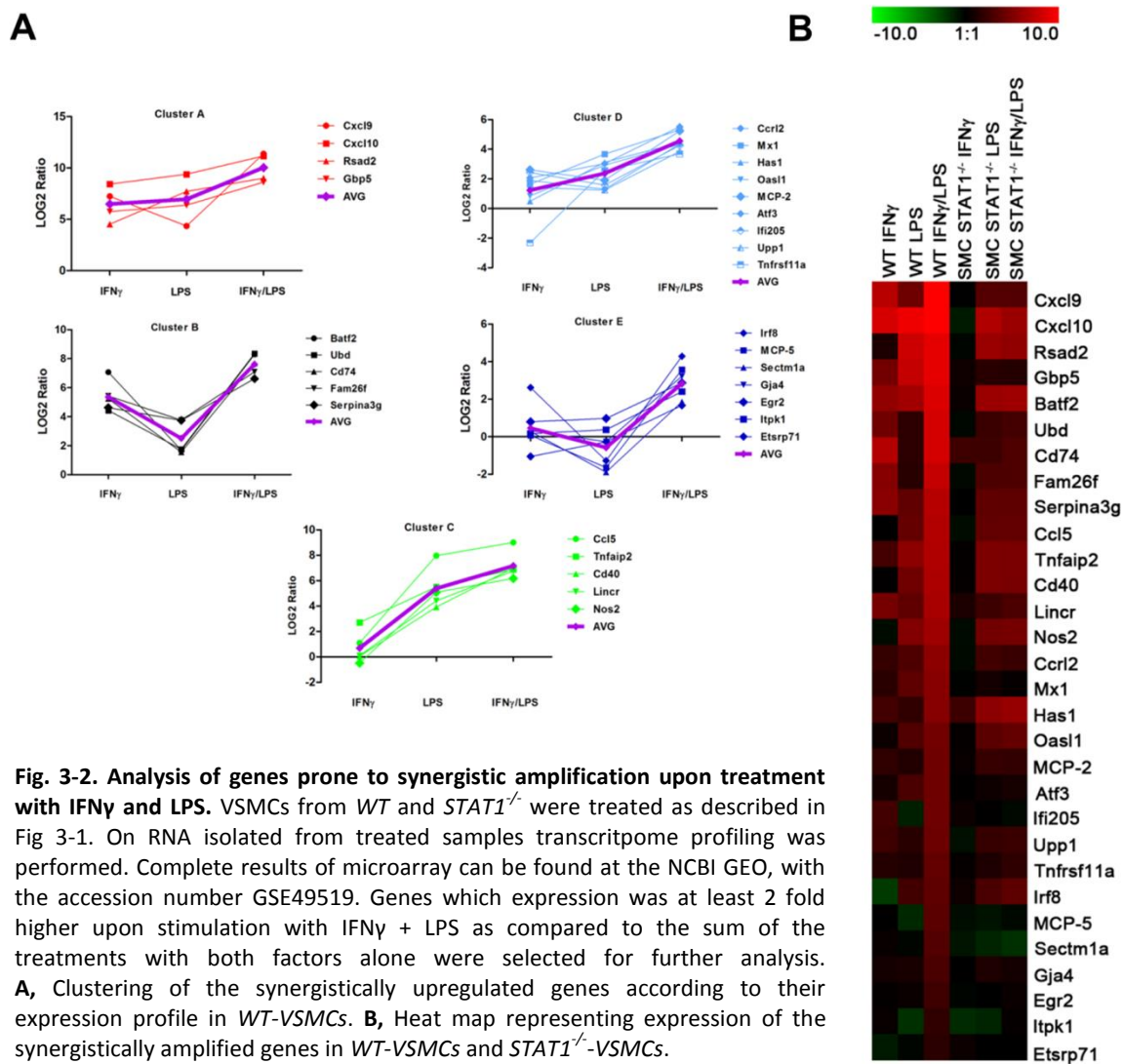
### Transcriptional responses in IFN $\gamma$ and LPS treated VSMCs depend on STAT1, NF $\kappa$ B and IRF and predict pro-atherogenic phenotype.

To examine how pretreatment of VSMCs with IFN $\gamma$  affected the response to LPS at a genome wide level, and in particular whether distinct sets of STAT1-dependent genes could be identified, we performed expression profiling. Complete results of microarray can be found at the NCBI GEO, with the accession number *GSE49519*. We aimed to identify genes that similar to *Cxcl10* and *iNOS* were synergistically affected by the interactions between IFN $\gamma$  and LPS. We selected genes which expression was at least 2 fold higher upon stimulation with IFN $\gamma$  + LPS as compared to the sum of the treatments with both factors alone.

**Table 3-3. Gene ontology classification of synergistically amplified genes**

Term ID	Description	Frequency	log <sub>10</sub> p-value	Uniqueness	Dispensability
GO:0051707	response to other organism	0.01	-10.10	0.56	0.00
GO:0009607	response to biotic stimulus	0.01	-9.62	0.66	0.40
GO:0006952	defense response	0.01	-8.91	0.63	0.41
GO:0002376	immune system process	0.01	-7.61	0.97	0.00
GO:0071345	cellular response to cytokine stimulus	0.00	-6.87	0.52	0.32
GO:0006950	response to stress	0.04	-6.59	0.61	0.50
GO:0006955	immune response	0.01	-6.26	0.41	0.39
GO:0006954	inflammatory response	0.00	-5.91	0.68	0.49
GO:0045071	negative regulation of viral genome replication	0.00	-5.04	0.76	0.47
GO:0009611	response to wounding	0.00	-4.93	0.68	0.53

Table 3-2 presents 30 genes prone to signal integration. Functional analysis of the genes listed in Table 3-2 revealed substantially enriched gene ontology terms that are related to biological functions involved in host defense, immune response, inflammatory response, cytokine response, response to stress and to wound healing (Table 3-3). Among them were genes involved in chemotaxis like *Cxcl9* (fifteen fold higher after combined treatment), *Cxcl10*, *Ccl5*, *Ccl12*, *Ccl8*, *Ccl12*, *Cxcl10*, adhesion molecules *Cd40*, *Cd74*, and the antiviral and antibacterial response genes *Irf8*, *Rsad2*, *Mx1*, *Oasl1*, *Gbp5*, *Nos2*, *Batf2* and *Tnfrsf11a*. Depending on their characteristic response pattern in *WT-VSMCs*, we could divide genes listed in Table 3-2 into five groups (Fig. 3-2).



**Fig. 3-2. Analysis of genes prone to synergistic amplification upon treatment with IFN $\gamma$  and LPS.** VSMCs from WT and STAT1 $^{-/-}$  were treated as described in Fig 3-1. On RNA isolated from treated samples transcriptome profiling was performed. Complete results of microarray can be found at the NCBI GEO, with the accession number GSE49519. Genes which expression was at least 2 fold higher upon stimulation with IFN $\gamma$  + LPS as compared to the sum of the treatments with both factors alone were selected for further analysis. **A**, Clustering of the synergistically upregulated genes according to their expression profile in WT-VSMCs. **B**, Heat map representing expression of the synergistically amplified genes in WT-VSMCs and STAT1 $^{-/-}$ -VSMCs.

First group consist of *Cxcl9*, *Cxcl10*, *Rsad2* and *Gbp5*. These genes were highly expressed upon treatment with IFN $\gamma$  and LPS alone and highly amplified upon combined treatment (Cluster A in Table 3-2 and Fig. 3-2 A). Genes form the second group (*Batf2*, *Ubd*, *Cd74*, *Fam26f* and *Serpina3g*) responded moderately to LPS, were highly expressed upon treatment with IFN $\gamma$  and synergistically amplified upon combined treatment (Cluster B in Table 3-2 and Fig. 3-2 A). Alike, genes from the third group responded moderately to IFN $\gamma$ , highly to LPS and were highly amplified upon combined treatment. This group was represented by *Ccl5*, *Tnfaip2*, *Cd40*, *Linc* and *Nos2* (Cluster C in Table 3-2 and Fig. 3-2 A). The fourth group of genes was represented by *Ccr12*, *Mx1*, *Has1*, *Oasl1*, *MCP-2*, *Atf3*, *Ifi205*, *Upp1* and *Tnfrsf11a* and displayed mild or no response to IFN $\gamma$ , mild response to LPS, and mild amplification after combined treatment

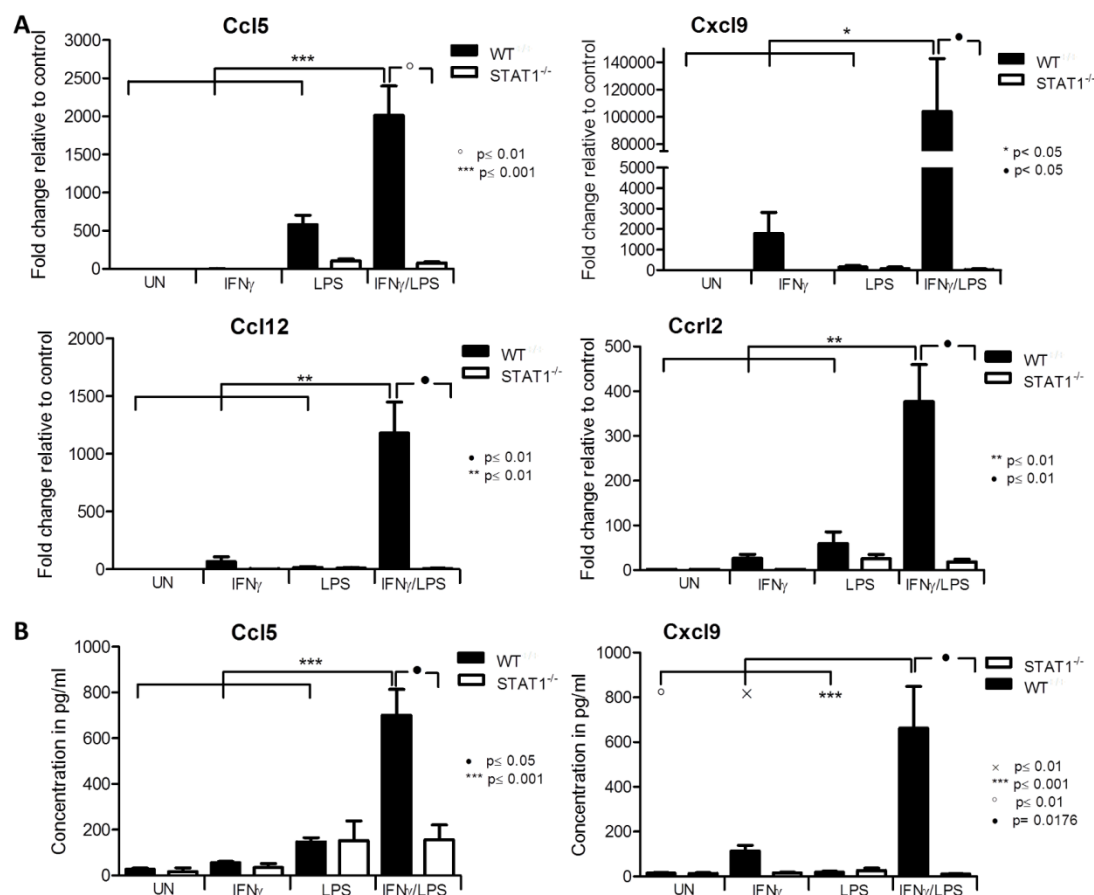


(Cluster D in Table 3-2 and Fig. 3-2 A). Finally, we could also identify genes which showed minor or no response to IFN $\gamma$  and LPS alone, but were highly amplified in expression after combined treatment [e.g., *Irf8*, *MCP-5*, *Sectm1a*, *Gja4*, *Egr2*, *Itpk1* and *Etsrp71*] (Cluster E (Cluster D in Table 3-2 and Fig. 3-2A). Next, we compared expression profile between WT-VSMCs and *STAT1*<sup>-/-</sup> VSMCs with respect to the genes listed in Table 3-2 (Fig. 3-2 B). The synergistic effect of combined treatment with IFN $\gamma$  and LPS was no longer present in *STAT1*-deficient VSMCs. As expected, lack of *STAT1* resulted in lack of response to IFN $\gamma$ , except *Mcp-2* (*Ccl8*) which expression seemed to be *STAT1* independent (Table 3-2, 6.12 vs. 5.12). Although effect of LPS stimulation was not completely abolished in *STAT1*<sup>-/-</sup> VSMCs, in 50% of the genes listed In Table 3-2 response to LPS was ameliorated. Only expression of *Mcp-2* had a different characteristic. *STAT1*-deficient VSMCs triggered accelerated expression of *Mcp-2* in response to LPS compared with WT-VSMCs (Table 3-2, 3.75 vs. 44.53).

Additionally, to provide *in silico* evidence that *STAT1* is involved in the regulation of above mentioned genes, we searched their promoter regions for overrepresented motifs that may be involved in the regulation of expression (Table 3-2). Promoter analysis of the synergistically upregulated genes predicted the presence of *STAT*-*NFkB* and *IRF*-*NFkB* modules or combinations of separate *ISRE*, *STAT* or *NFkB* binding sites, strongly suggesting the cooperative involvement of *NFkB*, *STAT1* and/or *IRFs* in the transcriptional regulation of all of these genes in response to IFN $\gamma$  and LPS.

### Signal integration between IFN $\gamma$ and LPS in VSMCs leads to increased migration of T-lymphocytes.

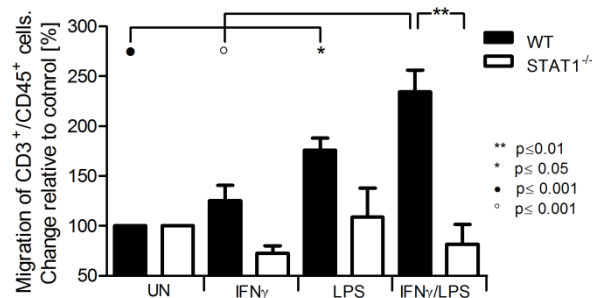
Subsequently, by performing qPCR and ELISA on selected genes involved in inflammatory processes, we aimed to confirm results obtained in microarray experiments (Fig. 3-3).



**Fig. 3-3. Effect of STAT1 dependent signal integration on chemokines expression.** VSMCs from WT and STAT1<sup>-/-</sup> were treated as described in Fig 3-1. **A**, RNA from VSMCs was isolated and qRT-PCR transcriptase PCR for *Ccl5*, *Cxcl9*, *Ccl12*, *Ccr12* using *Gapdh* as internal control was performed. **B**, On the medium remained after treatment of VSMCs ELISA for Ccl5 and Cxcl9 was performed. Data represent means of at least 3 independent biological experiments  $\pm$ SEM and  $p < 0.05$  was considered as significant.

Indeed, combined treatment led to amplification of *Ccl5*, *Cxcl9*, *Ccl12*, *Ccr12* only in WT-VSMCs as compared to stimulation with IFN $\gamma$  and LPS alone (Fig. 3-3A). Analysis of the proteins in the medium, reflected results obtained at mRNA level (Fig. 3-3B). In contrast response to stimulation in STAT1<sup>-/-</sup> VSMCs was abolished (Fig. 3-3). Next, we analyzed whether synergistic amplification of the genes has functional consequence. Many of the chemokines listed in Table 3-2 are known to be involved in chemotaxis of T-lymphocytes [193].

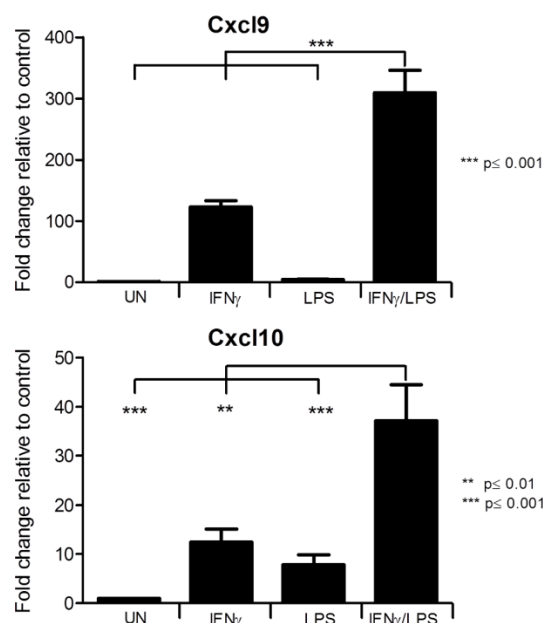
Thus, we examined the effect of IFN $\gamma$  and LPS crosstalk in T-cell trafficking towards conditioned medium (Fig. 3-4). While the migration of CD3 $^{+}$ /CD45 $^{+}$  cells towards medium of WT-VSMCs treated with IFN $\gamma$  and LPS alone led to increase of 125% and 175% respectively, treatment with both IFN $\gamma$  and LPS was 234%. As expected, the chemotactic response of splenocytes towards the conditioned medium obtained after treatment of *STAT1* $^{-/-}$ -VSMCs was highly attenuated (Fig. 3-4).



**Fig. 3-4. Amplification of chemokines expression leads to increased splenocytes migration.** Migration assay of CD45 $^{+}$ /CD3 $^{+}$  performed on conditioned medium remained after treatment of VSMCs WT and *STAT1* $^{-/-}$ . Data represent means of at least 3 independent biological experiments  $\pm$ SEM and  $p < 0.05$  was considered as significant.

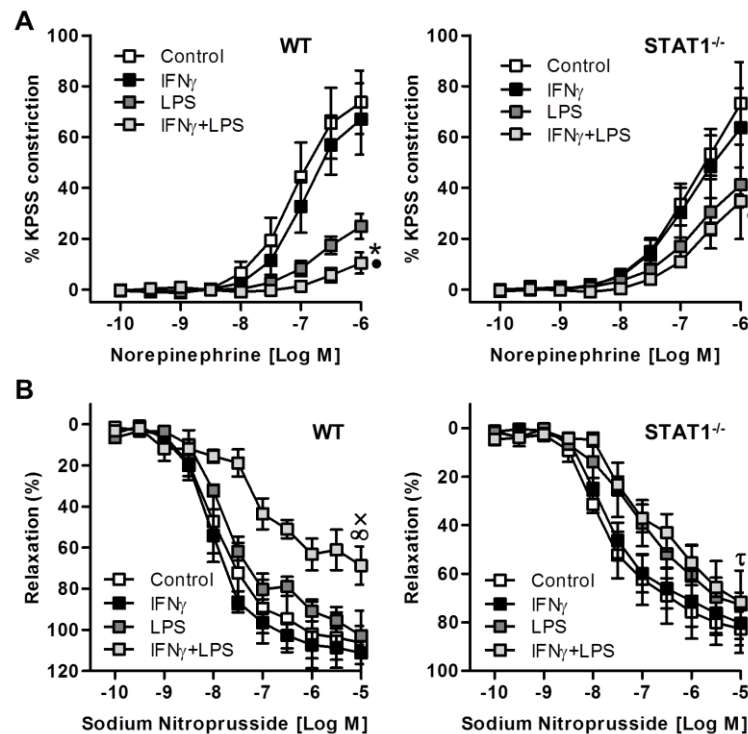
### Signal integration between IFN $\gamma$ and LPS in aortic rings leads to abolished response to norepinephrine and sodium nitroprusside.

To further confirm functional relevance of concomitant IFN $\gamma$  and LPS stimulation, we verified expression of *Cxcl9* and *Cxcl10* in *ex vivo* stimulated aortas isolated from WT animals. Indeed stimulation with IFN $\gamma$  followed by LPS led to synergistic amplification of *Cxcl9* and *Cxcl10* (Fig. 3-5).



**Fig. 3-5. Cxcl9 and Cxcl10 overexpression upon IFN $\gamma$  and LPS stimulation in WT aortas.** Isolated aortas from WT animals were cleaned from perivascular fat and placed in DMEM medium with 2% FBS. Next aortas were stimulated as in Fig. 3-1. RNA was isolated and qRT-PCR for *Cxcl9* and *Cxcl10* using *Gapdh* as internal control was performed. Data represent means of 3 experiments  $\pm$ SEM and  $p < 0.05$  was considered as significant.

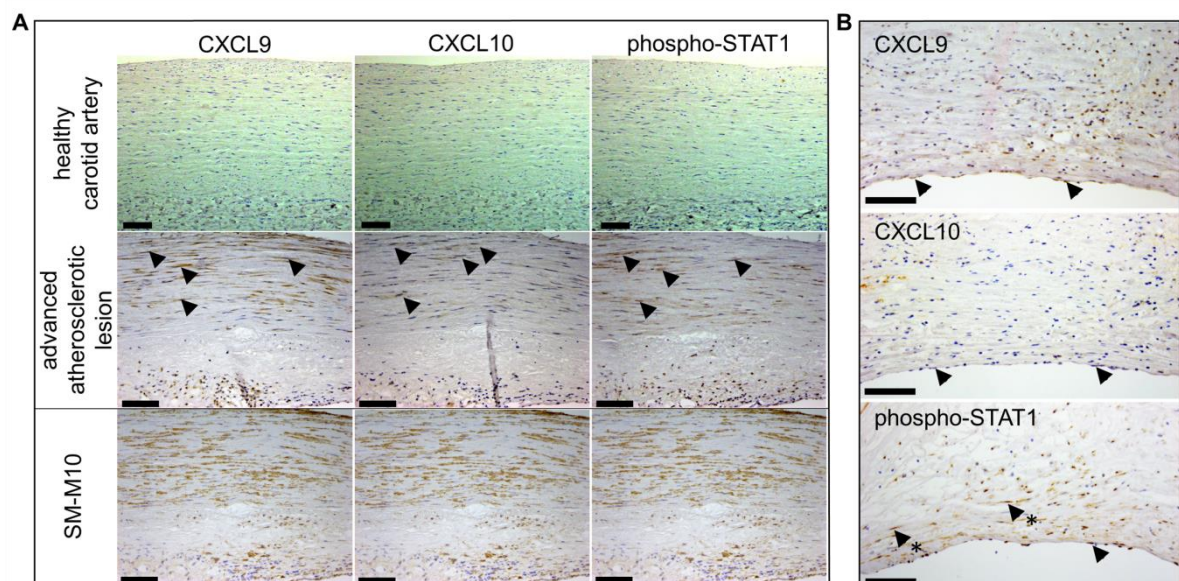
Among the genes that were highly amplified upon treatment with IFN $\gamma$  and LPS was inducible nitric oxide synthase (*iNOS*, *Nos2*). Activity of iNOS was found to be crucial in regulating vessel function. Thus, to further evaluate physiological ramifications of our experimental conditions, we tested vessel function using myograph (Fig. 3-6). While treatment with IFN $\gamma$  did not result in a significant change of the response neither to norepinephrine nor sodium nitroprusside, stimulation with LPS resulted in reduced response to norepinephrine in WT but also STAT1 $^{-/-}$ . Moreover, treatment of the WT aortic rings with IFN $\gamma$  followed by LPS, resulted in ameliorated contractile response to norepinephrine and dilator response to sodium nitroprusside. In contrast aortic rings isolated from STAT1 null mice did not reveal ameliorated response to noradrenaline and sodium nitroprusside as compared to LPS stimulated vessel.



**Fig. 3-6. Abolished response to norepinephrine and sodium nitroprusside in aortic rings stimulated with IFN $\gamma$  and LPS.** Isolated aortic rings from WT and STAT1 $^{-/-}$  mice were incubated with 10ng/ml IFN $\gamma$  for 8h or with 1 $\mu$ g/ml of LPS for 4h or with IFN $\gamma$  for 4h followed by IFN $\gamma$  and LPS for additional 4h. Next, response to norepinephrine and sodium nitroprusside was tested on the wire myograph. **A**, Response to noradrenaline in WT and STAT1-deficient mice presented as a percentage of maximal constriction to KPSS. \* $p < 0.001$  vs. WT control; • $p < 0.001$  vs. WT LPS;  $\circ p < 0.001$  vs. STAT1 $^{-/-}$  control. **B**, Response to stepwise increased concentration of sodium nitroprusside. x $p < 0.05$  vs. WT control;  $\infty p < 0.01$  vs. WT LPS;  $\tau p < 0.05$  STAT1 $^{-/-}$  control. Aortas isolated from 3-4 animals per group were taken. Two-way ANOVA test with Bonferroni post hoc test was used.

### STAT1 activation and CXCL9 and CXCL10 expression in ECs and VSMCs from human carotid atherosclerotic plaques.

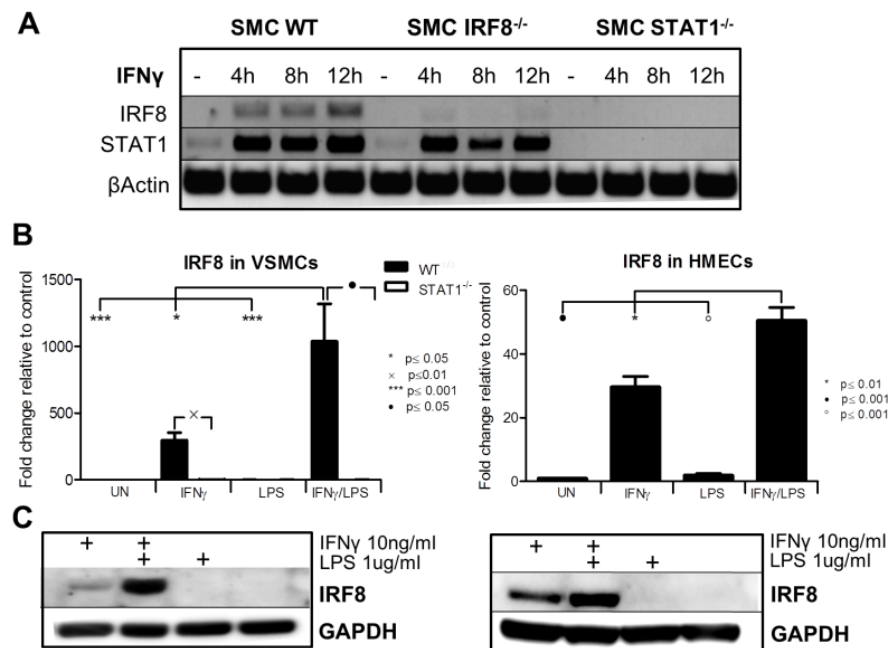
To gain further insight into the role of STAT1 in atherosclerosis, we performed immunohistochemistry staining for phosphorylated STAT1 as well as STAT1-dependent chemokines, CXCL9 and CXCL10 in human advanced atherosclerotic plaques of carotid arteries. As it is shown in Fig. 3-7 neither phosphorylated STAT1 nor CXCL9, nor CXCL10 were present in carotid arteries from the control group (Fig. 3-7 A, upper row). In contrast, VSMCs in the lesions highly expressed phosphorylated STAT1 which was associated with expression of chemokine CXCL9 and to a lesser extent CXCL10 (Fig. 3-7 A, middle row). Additionally, ECs covering the plaque likewise showed predominant staining for phosphorylated STAT1 associated with CXCL9, and to a lesser extent with CXCL10 staining. This result gives an additional proof for a pro-atherogenic role of STAT1 in vascular cells of atherosclerotic plaques.



**Fig. 3-7. Expression of pSTAT1, CXCL9, CXCL10 in human atherosclerotic lesions *in situ*.** Staining of the sections prepared from normal human artery exhibited no presence of pSTAT1, CXCL9, CXCL10 (A, upper panel). In contrast, all three proteins could be detected in SM-M10 positive cells in atherosclerotic plaques (A, middle panel) as well as in the endothelial cells at the lumen side (B). A representative analysis is shown of 6 human carotid atherosclerotic lesions and 4 healthy controls. Arrows represent examples of positive staining. In B arrows with asterix indicate an examples of positively stained VSMCs. Scale bar = 100µm.

### IRF8 is highly expressed in SMCs and ECs in response to IFN $\gamma$ and LPS.

Among, the genes which were synergistically amplified upon combined treatment with IFN $\gamma$  and LPS was also IRF8. This transcription factor is considered to be expressed only in lymphoid-cell lineages such as B, T, dendritic cells and macrophages [82]. According to microarray results, expression of IRF8 was IFN $\gamma$  dependent and highly upregulated upon treatment with IFN $\gamma$  and LPS (Tab 3-2). Treatment with LPS alone did not affect expression of IRF8. To support our genome-wide studies, we treated VSMCs isolated from WT, STAT1 and IRF8 deficient mice with IFN $\gamma$ . We observed time dependent upregulation of IRF8 only in *WT*-VSMCs. Expression of IRF8 was not present either in *IRF8*<sup>-/-</sup> or *STAT1*<sup>-/-</sup>-VSMCs (Fig. 3-8 A). Next, we verified susceptibility of IRF8 to signal integration in cells from the vasculature. IRF8 was not present in basal condition in *WT*-VSMCs and HMECs (Fig. 3-8 B).

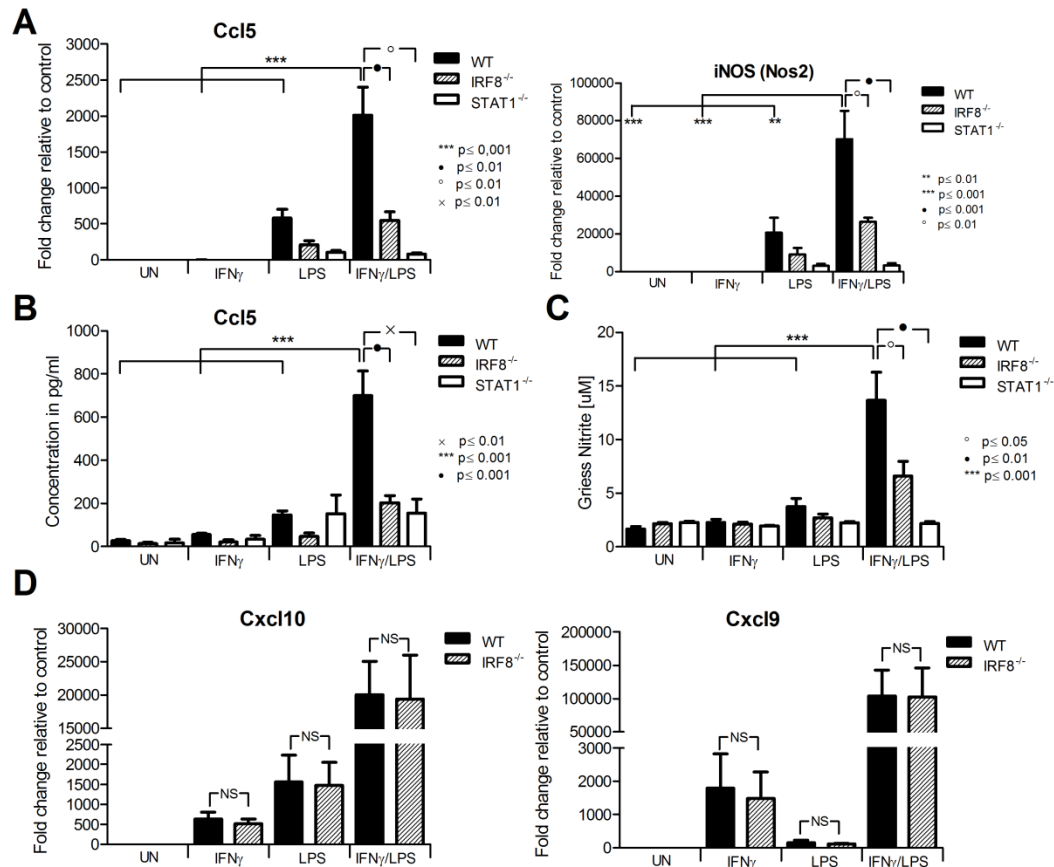


**Fig. 3-8. IRF8 is expressed in the vasculature.** **A**, VSMCs from *WT*, *STAT1*<sup>-/-</sup>, and *IRF8*<sup>-/-</sup> were treated with IFN $\gamma$  for indicated time points. RNA was isolated and subjected to PCR for *IRF8* using  $\beta$ Actin as internal control. **B**, Cells were treated as in Fig 3-1. qRT-PCR for *IRF8*, using *GAPDH* as internal control was performed in VSMCs (left panel) and HMECs (right panel). Data represent means of at least 3 independent experiments  $\pm$ SEM and  $p < 0.05$  was considered as significant. **C**, Representative Blot for IRF8. Protein extracts from treated cells were analyzed for IRF8 and GAPDH. Left panel presents data obtained from VSMCs and right from HMECs.

Only treatment with IFN $\gamma$  led to the upregulation of IRF8 in *WT-VSMCs* and HMECs. Moreover, pretreatment of VSMCs and HMECs with IFN $\gamma$  for 4h followed by LPS for another 4h resulted in amplification of IRF8 expression. IRF8 was not present in *STAT1*<sup>-/-</sup>-VSMCs indicating STAT1 dependent expression of IRF8. In analogy to the mRNA results, Western experiment confirmed upregulation of IRF8 upon treatment with IFN $\gamma$  and amplification of its expression in the presence of both IFN $\gamma$  and LPS in HMECs and *WT-VSMCs* but not *STAT1*<sup>-/-</sup> (Fig. 3-8 C). This result indicates STAT1 dependent expression of IRF8 upon stimulation with IFN $\gamma$  and confirms amplification of IRF8 upon stimulation with IFN $\gamma$  and LPS.

#### **IRF8 mediates IFN $\gamma$ and LPS induced gene expression in vascular cells.**

IRF8 is an important transcription factor which regulates not only immune cells development but also their function and is associated with expression of several proinflammatory genes [82, 194]. Therefore, to further characterize the role of IRF8 in the vasculature, we evaluated expression patterns of *Ccl5* and *Nos2*. Literature data indicates that expression of above mentioned genes in immune cells is IRF8-dependent [195].



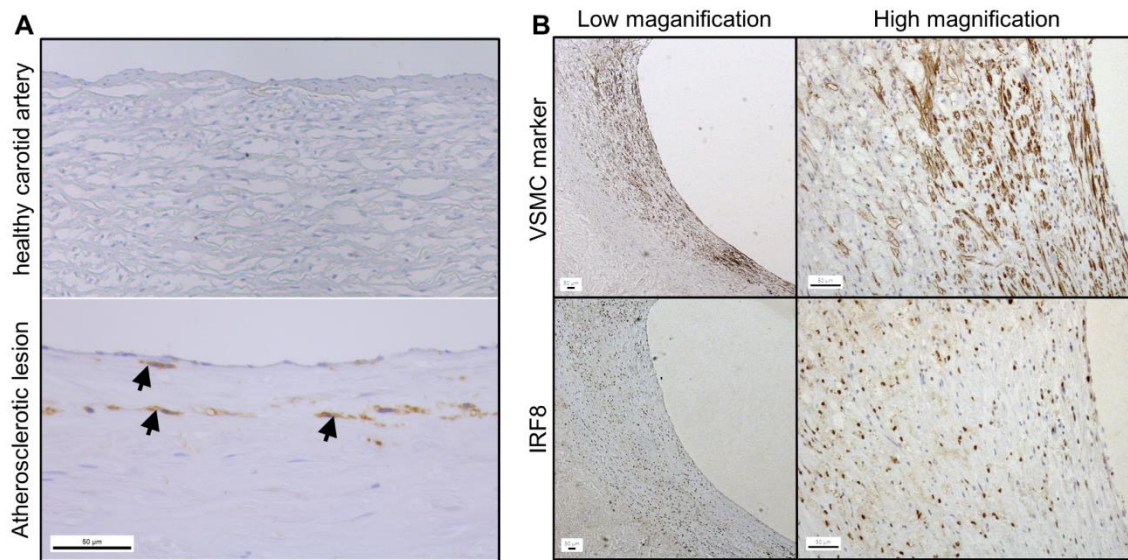
**Fig. 3-9. IRF8 participates in a crosstalk between IFN $\gamma$  and LPS by regulating expression of *Ccl5* and *iNOS* but not *Cxcl10* and *Cxcl9*.** VSMCs from WT and STAT1 $^{-/-}$ , IRF8 $^{-/-}$  were treated as described in Fig 1. RNA was isolated and qRT-PCR for *Ccl5*, *iNOS* (A) and *Cxcl10*, *Cxcl9* (C) using *Gapdh* as internal control was performed. B, On the medium remained after treatment ELISA for *Ccl5* was performed. C, After treatment medium was refreshed and cells were cultivated for further 24h. 100 $\mu$ l was used to measure nitrite. Data represent means of at least 3 independent biological experiments  $\pm$ SEM and p<0.05 was considered as significant. NS – not significant

Expression patterns were evaluated by qPCR and compared with WT and STAT1 $^{-/-}$  cells (Fig. 3-9). Indeed the IRF8 dependent regulation of *Ccl5* and *Nos2* on mRNA and on protein level (Fig. 3-9 A and B) was observed. Treatment with IFN $\gamma$  and LPS led to amplification of *Ccl5* and *Nos2* in WT and IRF8 $^{-/-}$  VSMCs. However, response to both stimuli was highly attenuated in IRF8 $^{-/-}$ -VSMCs compared with WT-VSMCs. Nitrite accumulation (Fig. 3-9 C) and Ccl5 expression (Fig. 3-9 B) measured in the medium confirmed that result. In contrast to *Ccl5* and *Nos2*, expression of *Cxcl10* and *Cxcl9* in response to IFN $\gamma$  and LPS in WT VSMCs was similar to that in IRF8 $^{-/-}$ -VSMCs (Fig. 3-9 D).



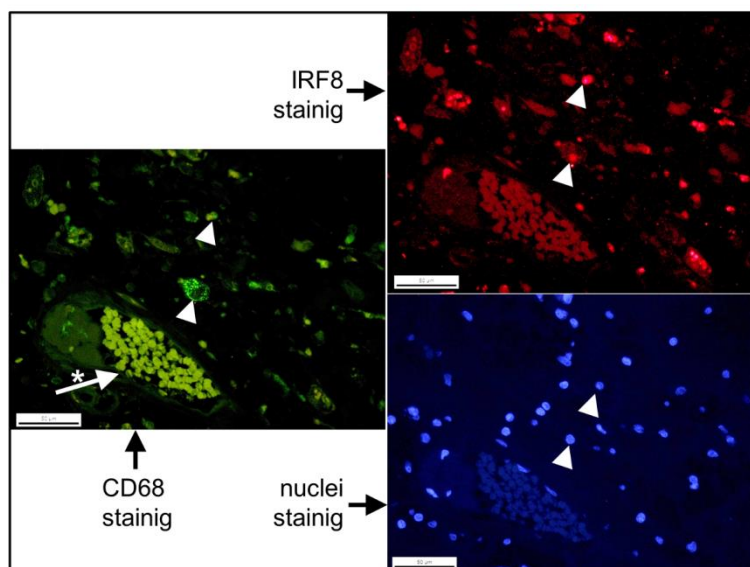
### IRF8 is expressed in ECs and VSMCs from human carotid atherosclerotic plaques.

To obtain further evidence for the expression of IRF8 in vascular cells in human tissue, we performed immunohistochemistry staining of IRF8 in human advanced atherosclerotic plaques of carotid arteries and compared to healthy vessels. While there was no positive IRF8 staining in control vessels (Fig. 3-10 A), human atherosclerotic lesions were positive for IRF8 and expressed in areas positive for SMCs (Fig. 3-10 B).



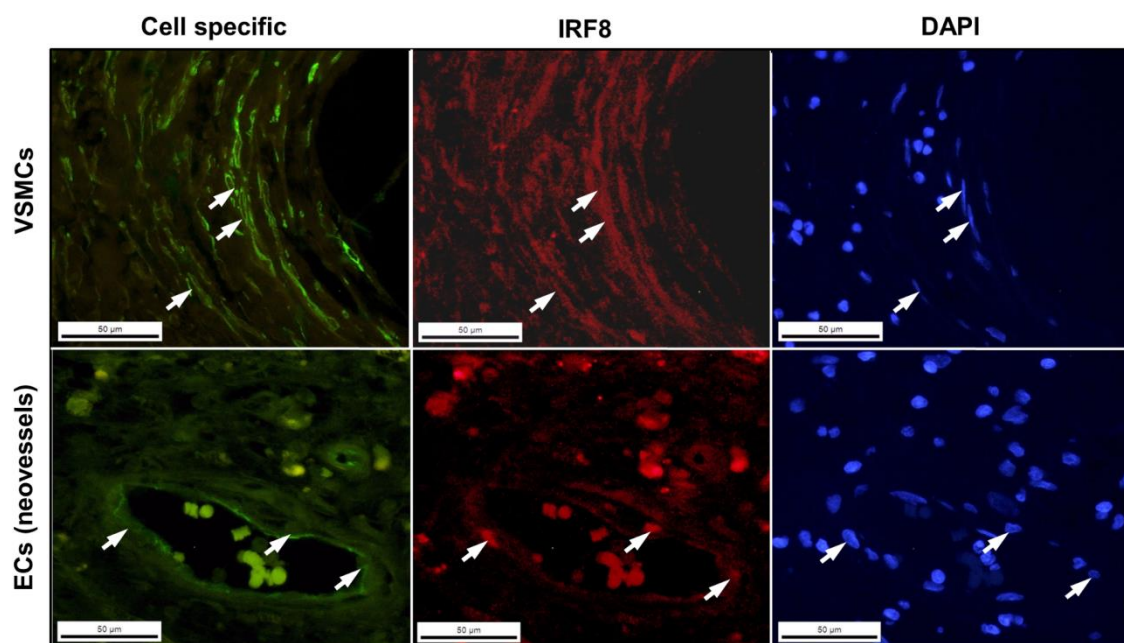
**Fig. 3-10. Expression of IRF8 in human atherosclerotic lesions.** Staining of the sections prepared from normal human artery exhibited no presence of IRF8 (A, upper panel). In contrast, IRF8 could be detected in cells that correlate with expression for SMC marker in atherosclerotic plaque. Arrows represent examples of positive staining.

To specify which cells express IRF8, we performed fluorescent immunohistochemistry. We identified strong expression of IRF8 in CD68 positive cells (Fig. 3-11).



**Fig. 3-11. Fluorescent staining of in advanced carotid atherosclerotic plaques for IRF8.** Abundance of IRF8 correlated with expression of macrophage marker (cells expressing CD68 marker). Combined staining with FITC (macrophage) and Cy3 (IRF8) fluorescence dye were used. Cell nuclei were counterstained with DAPI.

In addition to macrophages (cells expressing CD68 marker), VSMCs seemed to be positive for this staining (Fig. 3-12 upper panel). However, the expression was very weak and not all cells were stained. Furthermore, ECs of intra-plaque neovessels were also positive for IRF8 (Fig. 3-12 lower panel). In contrast, ECs covering the plaque showed negative staining (data not shown). Presented results indicate that IRF8 is expressed in human carotid plaques among inflammatory as well as non-inflammatory cells. However, these results are still not unambiguous and further experiments are necessary to confirm the presence of IRF8 in atheromata.



**Fig. 3-12. Staining for IRF8 in human carotid plaques.** Selected examples of fluorescent staining of VSMCs and ECs within neovessels in advanced carotid atherosclerotic plaques for IRF8. A combined staining with FITC (green, cells specific) and Cy3 (red, IRF8) fluorescence dye were used. Cell nuclei were counterstained with DAPI.

## Discussion

ECs and VSMCs sustain the blood flow and regulate the vascular tone, thus they play a pivotal role in maintaining homeostasis of the cardiovascular system. Nevertheless, in the presence of cardiovascular risk factors the cells that form the vessel wall are activated and demonstrate a phenotype that is characteristic of the host defense response. This change of the phenotype

results in the expression of proinflammatory genes such as cytokines and chemokines and is fundamental in the pathophysiology of many disorders, including atherosclerosis [196, 197].

In a previous chapter we showed that signal integration between important contributors of the inflammation is also present in non-immune cells. Our results suggested that in ECs and VSMCs, STAT1 creates a platform for crosstalk between IFN $\gamma$  and TLR4 and thus triggers expression of proinflammatory genes such as *Cxcl10*. Here we present results which clearly confirm previous findings. Signal integration between both pathways led to high expression of *Cxcl10* and *iNOS*, which was TLR4- and STAT1-dependent (Fig. 3-1). We carried out transcription profiling to further investigate how signal integration between IFN $\gamma$  and TLR4 modulates gene expression and, consequently, how this alteration affects vascular function. We identified 30 STAT1-dependent genes whose expression upon combined treatment was at least two-fold higher as compared to treatment with IFN $\gamma$  and LPS alone. Functional analysis as presented in Table 3-3 revealed that these genes are involved in a number of biological processes related to inflammation, stress and wound healing. These included chemokines *Cxcl9*, *Ccl12*, *Ccl8*, *Ccr12*, *Cxcl10* and *Ccl5*, adhesion molecules (*Cd40*, *Cd74*), and the antiviral and antibacterial response genes *Irf8*, *Rsad2*, *Mx1*, *Oasl*, *Gbp5*, *Nos2*, *Batf2* and *Tnfrsf11a*. Interestingly, meta-analysis performed in our laboratory identified the expression of a subset of genes in human plaques from the carotid and coronary artery [198]. Some of these genes, including *CXCL9*, *CXCL10*, *CCL5*, *CCL8*, *CRCL2*, *CD74* and *IRF8*, have previously been implicated in atherosclerosis [89, 199]. However, other genes were not, such as *GBP5*, *UBD*, *SECTM1*, *IFI16*, *UPP1* and *FAM26F*, and could therefore represent the potential novel biomarkers of atherosclerosis.

Depending on the characteristic response pattern in *WT-VSMCs*, we could divide the genes listed in Table 3-2 into five groups (Fig. 3-2). Next we verified the expression pattern of synergistically amplified genes in *STAT1*<sup>-/-</sup>-*VSMCs*. As was expected, a lack of STAT1 resulted in an abolished IFN $\gamma$  response and in deprivation of signal integration upon combined treatment (Fig. 3-2 B). With regard to the LPS response, a lack of STAT1 resulted in ameliorated expression in 50% of the genes listed in Table 3-2. ELISA and qPCR, which was used to additionally determine the expression profile of selected genes, validated our microarray

results (Fig. 3-3). Promoter analysis confirmed the presence of a variety of cis-regulatory elements such as STAT-NFκB and IRF-NFκB modules, or combinations of separate ISRE-, STAT- or NFκB-binding sites. Most of the genes listed in Table 3-2 do not have a unique cis-regulatory element, which strongly suggests the cooperative involvement of distinct transcription factors including NFκB, STAT1 and/or IRF in the transcriptional regulation of all of these genes in response to IFNγ and LPS. These results are in line with several studies that were performed in immune cells where the cooperative action of STAT1 and NFκB was related to gene expression in response to stimuli such as IFNγ and TNFα, IL-1β or LPS [132, 135-137, 139, 143]. For example, it was recently reported that IFNγ and TNFα synergistically regulated the transcription of many inflammatory genes [135-137, 139] including *CXCL9* [131], where independent interaction of STAT1 and NFκB was sufficient to mediate the transcriptional synergy [139]. Similar phenomena were observed for genes containing ISRE and NFκB elements. In this respect the NFκB motif in the *GBP1* promoter was required for a transcriptional response to TNFα and IL-1β in cooperation with IRF1-binding ISRE [200]. A similar synergistic effect of TNFα with IFNγ and LPS with IFNγ was observed on the promoter activity of several other genes, such as *ICAM-1* [201], *NOS2* [202], *CXCL10* or *CCL5* [137, 203]. Our results strongly suggest that a mechanism of synergistic amplification, primarily described in immune cells and based on cooperation between NFκB and STAT1 or NFκB and IRF1, is also present in cells from the vasculature [185]. Because there were no specific *cis*-elements that could explain the differences in the expression patterns of these 30 genes, we suggest that the affinity of the different transcription factors and their interplay most likely determines the transcriptional response of a particular gene.

A large group of genes listed in Table 3-2 belongs to the family of chemokines. This group of genes mediates chemotaxis of immune CD3<sup>+</sup>/CD45<sup>+</sup> cells and, consequently, directs them towards the inflammation site. We performed migration assay (Fig. 3-4) to verify whether synergistic amplification of genes related to chemotaxis has a functional consequence. Indeed, we observed that increased migration of T-cells towards the medium remained after treatment of *WT-VSMCs* with IFNγ and LPS as compared to the conditioned medium from cells treated with each factor alone. In contrast, migration towards the medium remained after treatment of *STAT1*<sup>-/-</sup>-*VSMCs* was attenuated. In addition, we confirmed amplification of *Cxcl9*

and *Cxcl10* in *ex vivo*-treated aortas isolated from WT mice (Fig. 3-5). Furthermore, the literature data indicate the involvement of many chemokines, including *CXCL9*, *CXCL10*, *CCL5*, *CCL8* and *CCRL2*, in leukocyte recruitment to the injured artery during vascular remodeling [193, 204, 205], and as such involvement in the pathogenesis of atherosclerosis. Therefore, to further confirm the results we performed IHC staining of atherosclerotic lesions isolated from human carotid arteries. In agreement with previously published data, we could confirm the presence of CXCL9 and CXCL10 in atherosclerotic lesions (Fig. 3-7). Importantly, we detected, for the first time, the presence of phosphorylated STAT1 which correlated with the expression of CXCL9 and CXCL10. These results, together with previously published data, point to the pro-atherogenic role of STAT1 in cells from the vasculature in human vascular disease [79]. Most importantly, signal integration between IFN $\gamma$  and LPS resulted not only in an increased abundance of chemokines but also contributed to vessel function by upregulation of *Nos2*. Inducible nitric oxide synthase has been found to promote vessel dysfunction and atherosclerosis [206]. Indeed, aortic rings isolated from WT treated by both stimuli had a highly ameliorated contractile response to norepinephrine and a dilator response to sodium nitroprusside (Fig. 3-6).

IRF8 was also among the genes that were synergistically amplified upon treatment with IFN $\gamma$  and LPS. This transcription factor was thought to be expressed only in immune cells. Our experiments clearly revealed its presence in VSMCs and HMECs at the mRNA as well as protein level (Fig. 3-8). However, expression of IRF8 was strictly regulated by IFN $\gamma$  and was not present at the basal condition. These results suggest that IRF8 is involved in the regulation of gene expression downstream of STAT1. Promoter analysis identified the presence of the regulatory element that was recognized by both STAT1 and NF $\kappa$ B in the IRF8 promoter (Table 3-2), which thus suggests the importance of the cooperation of STAT1 and NF $\kappa$ B in the amplification of IRF8 expression. Consequently, IRF8 can be considered as an additional platform for the regulation of crosstalk between IFN $\gamma$  and LPS in vascular cells. Interestingly, some evidence for IRF8's role in this crosstalk exists in immune cells. Zhao et al. showed that the synergistic induction of pro-inflammatory genes, such as *IL1*, *IL6*, *IL12* and *TNF $\alpha$* , is IRF8-dependent in macrophages [115]. Moreover, recent data pointed to the role of IRF8 in TLR4-mediated NF $\kappa$ B activation [207]. Indeed, our experiments performed in ECs and VSMCs

identified *Ccl5* and *iNOS* (but not *Cxcl9* and *Cxcl10*) as potential IRF8 targets (Fig. 3-9). These data are in line with results obtained in macrophages where cooperation of IRF8 with IRF1 and NFκB was essential for the IFNγ and LPS response. While interaction of IRF8 with IRF1 facilitated the response to IFNγ, the response to LPS was mediated by interaction between IRF8 and NFκB at the promoter site [115, 144, 208]. We hypothesize that a similar mechanism of interaction between IRF8 and other transcription factors regulates the expression of genes involved in inflammation, such as *Ccl5* and *Nos2*, which play a crucial role in atherogenic processes [209]. Indeed, *in silico* promoter analysis predicted the presence of an IRF-NFκB module in *Ccl5* and *Nos2* promoters. On the other hand, the promoters of both genes also contain a potential STAT1-NFκB module, which suggests the additional involvement of STAT1 as well.

Finally, we performed IHC staining of the material isolated from arteries affected with atherosclerosis to study whether IRF8 is expressed in the vessel wall. While expression of IRF8 was not detected in the control material, IRF8 expression could be detected in infiltrating macrophages and in VSMCs and ECs in the atherosclerotic plaque (Figs. 3-10-12). However, the expression in vascular cells seemed weaker and not all of these cells were positive for IRF8; thus further research will be required to prove the expression of IRF8 in atherosclerotic lesions. Nevertheless, our IHC results correlate with the fact that IRF8 expression is not detected in vascular cells but is completely dependent on pro-atherogenic stimuli, such as IFNγ and LPS, whereas a constitutive IRF8 expression pattern is present in immune cells [82].

In conclusion, our results indicate that in ECs and VSMCs, STAT1 and IRF8 together with the cooperation of other transcription factors such as IRF1 and NFκB orchestrate a platform for crosstalk between IFNγ and TLR4. Consequently, STAT1- and IRF8-mediated signal integration leads to synergistic amplification of genes involved in several proinflammatory processes such as chemotaxis, migration and oxidative stress. Because these processes are involved in the development and progression of atherosclerosis, STAT1 and IRF8 together with their downstream genes could represent potential targets of therapeutic intervention.

## Chapter 4

### Signal Transducer and Activator of Transcription protein (STAT)-1 in Angiotensin II-induced hypertensive organ damage

#### Introduction

Hypertension is one of the major risk factors for cardiovascular mortality and morbidity [210]. In Poland, prevalence of hypertension was reported in 32% of the adult population [211]. Among them only one third was aware of the disease and etiology of the disease remained unknown in 95% of the cases [211]. Angiotensin (Ang) II, a crucial mediator of the renin-angiotensin system, not only regulates the vascular tone but also induces inflammation and contributes to end organ damage. Ang II-induced hypertension causes cardiac remodeling characterized by inflammation, fibrosis and hypertrophy [212]. Genetically modified mice with kidney specific elevation of Ang II, have elevated inflammation and kidney fibrosis [213]. Although the exact mechanism of immune system sensitization remains unknown, it has been observed that Ang II stimulates the expression of chemokines (Ccl2, Cxcl10), cytokines (Il6 or TNF $\alpha$ ), and adhesion molecules (VCAM) [214-217]. Ang II can also act on monocyte differentiation and T cell function [31]. Treatment with angiotensin-converting enzyme (ACE) inhibitors and Ang II receptor blockers not only lowers the blood pressure but also diminishes the expression of adhesion molecules and decreases the number of adhered leukocytes [218-220]. Recent studies indicate that at least partially the function of Ang II is mediated through the activation of lymphocytes and subsequent IFN $\gamma$  secretion [73, 74, 221]. IFN $\gamma$  or IFN $\gamma$  receptor-deficient mice revealed not only improved vessel but also cardiac function, reduced inflammation and heart fibrosis despite Ang II infusion. The action of IFN $\gamma$  is mediated through the JAK/STAT pathway [118]. Stimulation with IFN $\gamma$  triggers conformational changes of the receptor and facilitates phosphorylation of the JAK1 and JAK2 kinases, which consequently phosphorylates transcription factor STAT1. This canonical mediator of IFN $\gamma$  signaling forms active dimers that trigger expression of STAT1-dependent genes such as *Cxcl10* and *Ccl2* [161, 222]. Importantly, not only IFN $\gamma$  but also Ang II can reveal its actions *via* activation of important components of the JAK/STAT pathway [223]. Ang II cooperates with the JAK2 kinase

to induce vessel contraction, and inhibition of JAK2 phosphorylation blocks blood pressure elevation [224, 225]. Similarly to IFN $\gamma$ , also Ang II contributes to activation of the JAK/STAT pathway, thus leading to the expression of nicotinamide adenine dinucleotide oxidase (NADPH) and, as a consequence, upregulation of ROS production [226, 227]. Mechanical stretch activates JAK/STAT *via* autocrine/paracrine-secreted Ang II [228].

Despite providing evidence for the importance of the JAK/STAT pathway in the regulation of Ang II response, surprisingly little is known about the role of transcription factor STAT1- and STAT1-dependent genes in such pathological settings. Although it has been shown that activated Ang II receptor I (AT1R) associates with the JAK2 kinase and triggers STAT1 phosphorylation, potential consequences of STAT1 activation are not fully understood [229, 230]. Both Ang II and STAT1 were identified to regulate autophagy, a catabolic process that degrades cytoplasmic components within the lysosome and is related to fibrosis and hypertrophy [231, 232].

Thus, to investigate the role of STAT1 in hypertension and hypertensive end-organ damage, we applied an Ang II-induced model of hypertension in STAT1-deficient mice. We hypothesized that activation of STAT1 during Ang II infusion upregulates chemokines, enhances chemotaxis and consequently results in heart fibrosis and vessel dysfunction.

## **Methods**

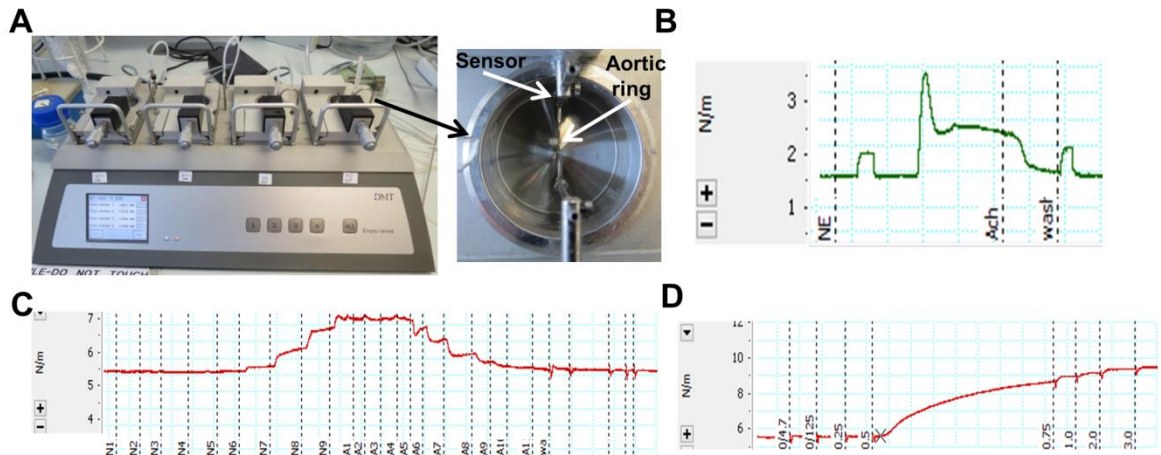
### **Animal experiments**

Mice wild-type C57BL/6 (WT) were obtained from Charles River Laboratories. STAT1<sup>-/-</sup> knockouts mice on C57BL/6 background were kindly provided by Thomas Decker. All strains were housed under controlled conditions of temperature (21°C) and were maintained on normal mouse chow diet and water *ad libitum*. All experiments performed in accordance with institutional guidelines. Angiotensin II (Sigma) in a concentration 1,5 $\mu$ g/g/day was infused using mini-osmotic pump (Alzet, model 2004) which was implanted subcutaneously under inhaled isoflurane anesthesia and buprenorphin. Blood pressure was measured using tail cuff pletysmography.



## Wire myograph and contractility studies

To measure circulatory function, descending thoracic aorta were cut into 2mm long rings and mounted in a 4-channel wire myograph (620M, Danish Myo Technology, Aarhus, Denmark) in the organ chamber filled with physiological saline solution (PSS) containing 118,99mM NaCl, 4,69mM KCl, 1,17mM  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 1,18mM  $\text{KH}_2\text{PO}_4$ , 2,5mM  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 25mM  $\text{NaHCO}_3$ , 0,03mM EDTA, 5,5mM Glucose (Fig. 4-1 A).



**Fig. 4-1. Protocol for the myograph experiment.** **A**, The wire myograph 620M (left picture) and the chamber with mounted aortic ring (right picture). **B**, On the isolated aortic rings, viability test with  $10^{-5}$  mol/L of noradrenaline (NE) followed by  $10^{-5}$  mol/L of acetylcholine was performed. **C**, Noradrenaline was used in stepwise increased, cumulative concentration ranging from  $10^{-11}$  to  $10^{-6}$  mol/L (N1-N9) and followed by acetylcholine dose-response curve from  $10^{-10}$  to  $10^{-5}$  mol/L (A1 to A11). **D**, Calcium-induced (receptor independent) vasoconstriction dose-response curve was performed. The myograph gives output readings as absolute tension generated elicited by vasoconstriction/vasodilatation in millinewton (mN).

During the experiment PSS buffer was aerated with carbogen (95%  $\text{O}_2$  + 5%  $\text{CO}_2$ ). After 20min of incubation (at  $37^\circ\text{C}$ ), calibration of the force transducer was performed [190]. Subsequently, vessels were pre-stretched to obtain optimal passive tension. Next, vascular functions were analyzed. Contractility was evaluated by substitution of PSS buffer for high potassium physiological saline solution (KPSS; 74,7mM NaCl, 60mM KCl, 1,17mM  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 1,18mM  $\text{KH}_2\text{PO}_4$ , 1,6mM  $\text{CaCl}_2$ , 14,9mM  $\text{NaHCO}_3$ , 0,026mM EDTA, 5,5mM Glucose). For testing viability, vessels were subjected to norepinephrine-induced constriction followed by acetylcholine dilation (Fig. 4-1 B). Activation of  $\alpha$ -adrenergic receptors by noradrenaline triggers the release of  $\text{Ca}^{+2}$  from the sarcoplasmic reticulum followed by membrane depolarization by activated chloride channels. This results in augmented

extracellular  $\text{Ca}^{+2}$  influx in the plasma membrane of the VSMCs and leads to an increase in intracellular  $\text{Ca}^{+2}$  concentration and vasoconstriction [233]. Opposite to norepinephrine, acetylcholine triggers endothelium-dependent vasodilatation *via* stimulation of muscarinic receptors. Acetylcholine leads to the release of nitric oxide from endothelium and causes opening of the potassium channels in the VSMCs thereby leading to hyperpolarization [234]. Action of acetylcholine is endothelium mediated and thus only vessels with intact endothelium fully respond to acetylcholine-mediated dilation. After washing out with PSS buffer and resting for 15 minutes, norepinephrine and acetylcholine dose-response curves were performed (Fig. 4-1 C). Noradrenaline was used in stepwise increased, cumulative concentration ranging from  $10^{-11}$  to  $10^{-6}$  mol/L (N1-N9), followed by acetylcholine dose-response curve from  $10^{-10}$  to  $10^{-5}$  mol/L (A1 to A11). Subsequently, vessels were washed with PSS buffer and left resting for 20 minutes. Finally, cells were washed with PSS buffer and calcium sensitivity was assessed by stepwise increases of calcium concentration (0 - 3mmol/L) in the organ bath under depolarizing conditions (125mmol/L potassium) starting at 0mmol/L calcium in the bath solution (Fig. 4-1 D).

### **RNA analysis**

Total RNA from kidney and heart was isolated using RNeasy Mini Kit (Qiagen). 10-20mg of the tissue was lysed using TissueLyser (Qiagen, 2x30Hz, 2min) in RLT buffer. Next, samples were homogenized using QIAshredder columns and RNA isolation was performed according to the manufacture's protocols. Isolated aortas were cleaned from perivascular fat and snap frozen on liquid nitrogen. Aorta was ground up with pestle and 1ml of Trizol was added. Total RNA from aorta was isolated using Trizol method followed by PureLink RNA kit (Life Technologies). cDNA was prepared using iScript cDNA synthesis kit (Bio-rad) following the manufacture's protocols. Quantitative real-time PCR was performed by using a MyiQ Real Time PCR detection system provided by Bio-rad. Forward and reverse primers are depicted in Table 4-1. The  $2^{-\text{ddCt}}$  method was applied for quantification [155]. Fold change in the target gene were normalized to *GAPDH* and relative to the mean expression at untreated sample. The results are expressed as fold of control from at least 3 independent assays.

**Table 4-1. List of primers used in chapter 4**

Gene Name	Forward	Reverse
<b>Gapdh</b>	TCGGTGTGAACGGATTGGC	TTTGGCTCCACCCTTCAAGTG
<b>MCP1 (CCL2)</b>	GCTGTAGTTTTTGTACCAAG	GATTACGGGTCAACTTCACA
<b>Nox4</b>	ACAGAAGGTCCCTAGCAGGAG	CAACAAACCACCTGAAACATGC
<b>Cxcl10</b>	TCATCCCTGCGAGCCTATCC	GGAGCCCTTTTAGACCTTTTT
<b>Cxcl9</b>	CTGCCATGAAGTCCGCTGTTCT	TCCCCCTCTTTTGCTTTTTCTT
<b>Col type 3A1</b>	CCTCAGACTTCTTTCCAGCCG	GTCTTGCTCCATCCCCAGTG
<b>iNOS (NOS2)</b>	TGGGGCAGTGGAGAGATTTT	TCTGGTCAAACCTCTTGGGGT
<b>p47phox</b>	AGAAGGCTGGGGAGGAGATA	TTCCGTTTGGTGCTCTCTGTG
<b>p22phox</b>	GCCCTCCACTTCCTGTTGTC	CCTCCTCTTCACCTCACTC
<b>NGAL</b>	CCAGGGCTGGCCAGTCACTC	TGGGTCTCTGCGCATCCCAGT <sup>[235]</sup>

### Western blot analysis

10-20mg of tissue was lysed using TissueLyser (Qiagen, 2x30Hz, 2min) in RIPA (Sigma) containing protease and phosphatase inhibitors (Roche). Protein concentrations were measured using BCA protein assay (Pierce). Protein extracts were heated with sample buffer (Life Technologies) containing dithiothreitol (90°C, 10min) and loaded on NuPAGE 4-12% Bis Tris Gel (Life Technologies). After electrophoresis (200V, 45min), proteins were transferred onto PVDV filter using wet transfer system (Bio-rad, 30V, 90mA, 16h at 4°C). Membranes were blocked with 5% BSA in TBS-Tween (TBST) and incubated with primary antibodies: rabbit monoclonal to LC3A/B antibody (overnight, 1:1000, Cell Signaling, 12741), or GAPDH (overnight, 1:15000, Cell Signaling, 5174). After washing in TBST for 30min at RT, membranes were incubated with secondary goat anti-rabbit antibody (1:15000, 30min at RT, Santa Cruz Biotechnology sc-2004). Next, membranes were washed in TBS for 30min and visualized using Luminata Forte or Luminata Classico (for GAPDH) Western HRP substrate (Millipore) in INTAS imaging system (Intas, Germany).

### Hydroxyproline measurement assay

Hydroxyproline was measured using Hydroxyproline assay provided by QuickZyme Biosciences. Briefly, approx. 20mg of the tissue were hydrolyzed in 6M HCl (Sigma) and incubated for 20h at 95°C together with provided standard. Samples were cooled to the RT,

centrifuged and transferred into new tube. Supernatants were diluted in 4M HCl and mixed with 75ul of the assay buffer and incubated 20min at RT. Next, 75ul of the detection reagent was added and incubated for 60min at 60°C in an oven. After incubation absorbance at 570nm was measured. Data were analyzed using MARS data analysis software (BMG Labtech).

### **Immunohistochemical analysis**

Part of the isolated tissue were fixed in 10% buffered formalin and embedded in paraffin. Sections (4 µm thick) were cut and stained for collagen via Picosirius red. For characterisation of the cells within isolated tissues, sections were treated with antibody for all nucleated hematopoietic cells (rat anti mouse CD45 BD Pharmingen 550539) dilution 1:50 overnight at RT. Following incubation with primary antibody visualisation was performed by peroxidase/DAB ChemMate Detection Kit according to the manufacturer's instructions.

### **Promoter Analysis**

Promoters for amplified STAT1 dependent genes were screened using GENOMATIX software (<http://www.genomatix.de/>). [160] The promoter regions from -1000 to +100bp were searched for binding sites (V\$IRF1.01 V\$ISGF3G.01 V\$ISRE.01 V\$ISRE.02 V\$CREL.01 V\$NFKAPPAB.01 V\$NFKAPPAB.02 V\$NFKAPPAB65.01 V\$STAT.01 V\$STAT1.01 V\$STAT1.02) or models with core similarity at least 0.85 (V\$STAT3.02 V\$STAT3.01).

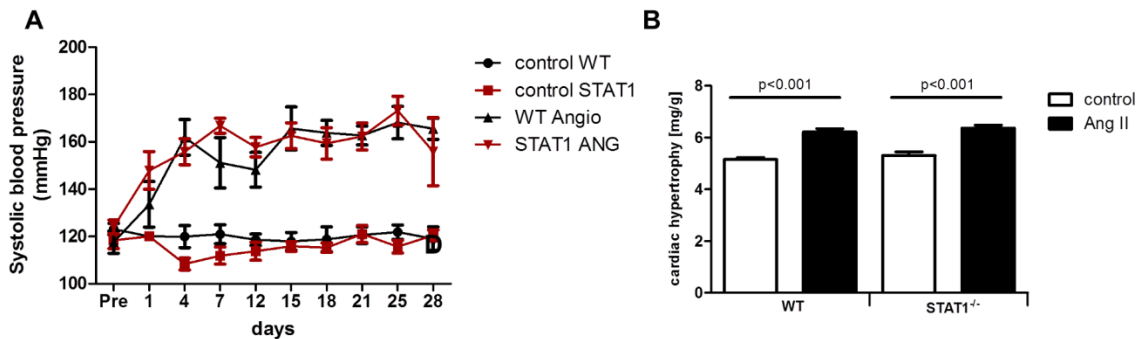
### **Statistical Analysis**

Data are presented as mean ± SEM. For comparisons between more than two groups one-way ANOVA with Tukey post-hoc test was used. In all other experiments comparing two groups, Student's t-test was used. In contractility studies, for comparison of maximum constriction among groups two-way ANOVA test with Bonferroni post hoc test was used. A probability value <0.05 was considered statistically significant (GraphPad Prism ® 5.0).

## **Results**

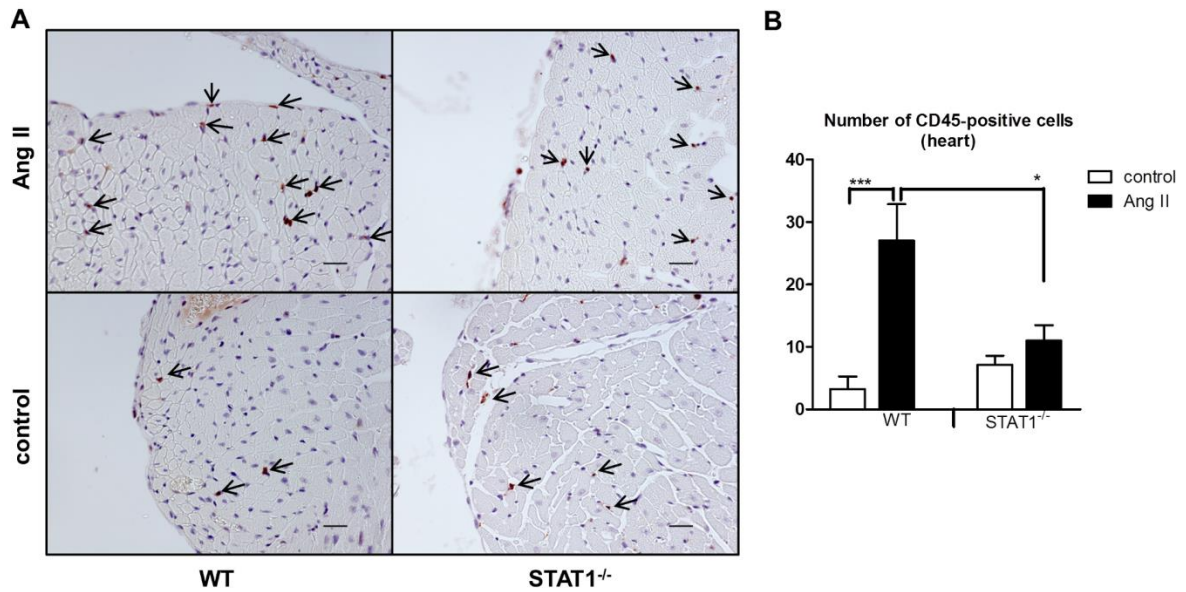
### Ang II-induced and STAT1 mediated cardiac damage

To characterize the role of STAT1 in hypertension and hypertensive induced end organ damage we subcutaneously infused Ang II in WT and STAT1<sup>-/-</sup> mice. Basal systolic blood pressure (BP) was the same among all groups and Ang II increased systolic BP in the same manner in WT and STAT1<sup>-/-</sup> (Fig. 4-2 A). Likewise, there was no difference in basal heart weight to body weight (HW/BW) ratio and both groups presented the same rate of cardiac hypertrophy upon stimulation with Ang II (Fig. 4-2 B).

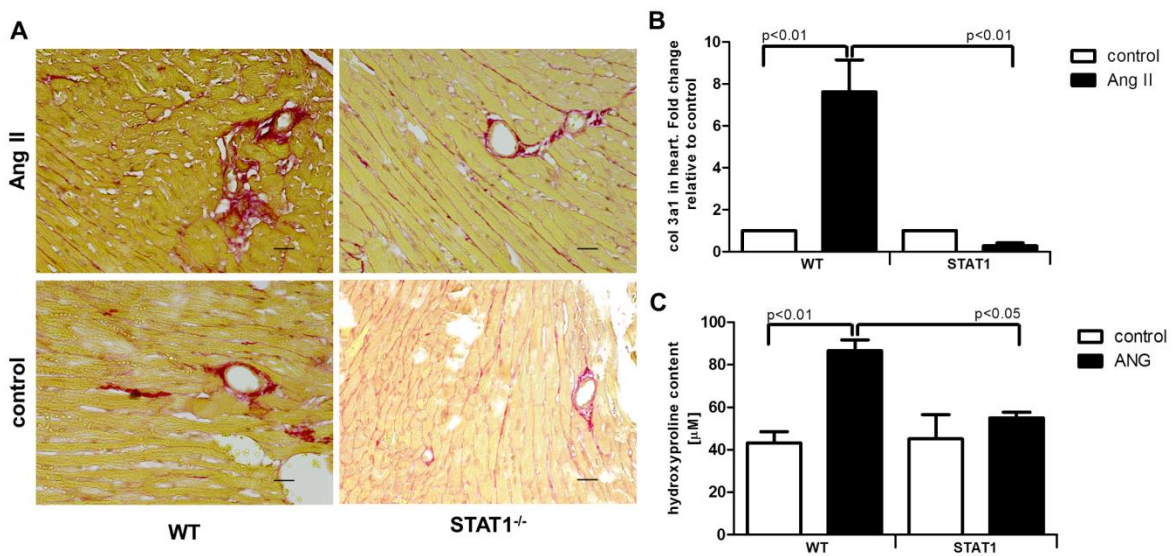


**Fig. 4-2. Role of STAT1 in hypertension and hypertensive induced end-organ damage.** C57BL/6 (WT) and STAT1<sup>-/-</sup> mice (C57BL/6 background) were treated for 28d with 1,5μg/g/day angiotensin II (Ang II), which was administered subcutaneously via osmotic minipump. **A**, Noninvasive blood pressure measurements obtained via the tail cuff method (n≥6 per group) **B**, Heart weight to body weight ratio (HW/BW) measured in Ang II treated STAT1 knockout vs WT mice.

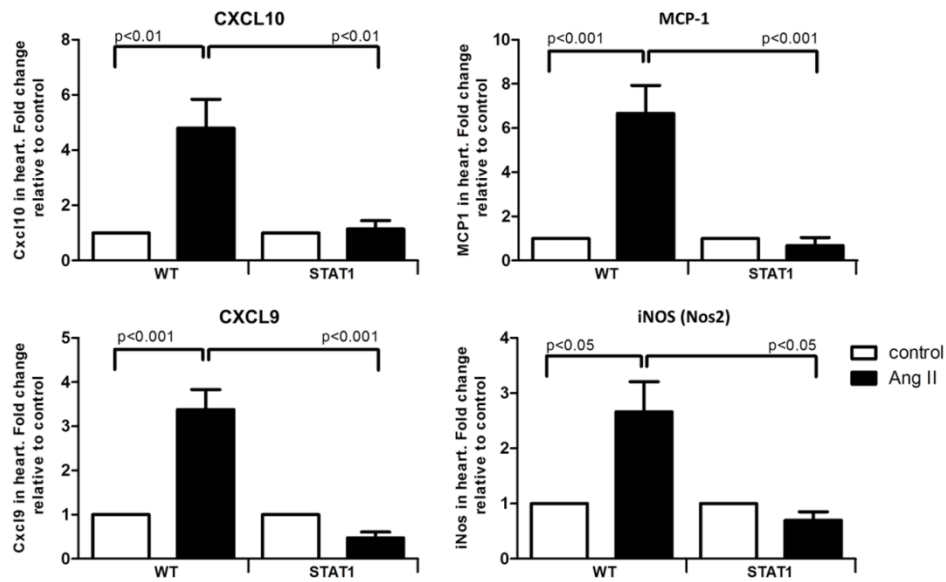
Next, we investigated the role of STAT1 in Ang II-induced inflammation of the heart. Staining for CD45<sup>+</sup> cells disclosed significantly increased infiltration of inflammatory cells in WT hearts which was markedly reduced in STAT1<sup>-/-</sup> mice upon chronic infusion of Ang II (Fig. 4-3). Sirius red staining demonstrated less perivascular fibrosis in STAT1<sup>-/-</sup> compared to WT treated with Ang II (Fig. 4-4 A). This result correlated with ameliorated expression of collagen in knockout mice (Fig. 4-4 B) and reduced tissue content of hydroxyproline in STAT1<sup>-/-</sup> exposed to Ang II (Fig. 4-4 C). Because STAT1 play an important role in the regulation of chemokine expression, we studied expression of these genes in heart tissue. Indeed, we observed increased expression of *Ccl2* (Mcp-1), *Cxcl9*, *Cxcl10* chemokines and marker of nitric oxide *iNos* only in hearts of stimulated WT animals (Fig. 4-5). Expression of inflammatory mediators correlated with the immune cell infiltration.



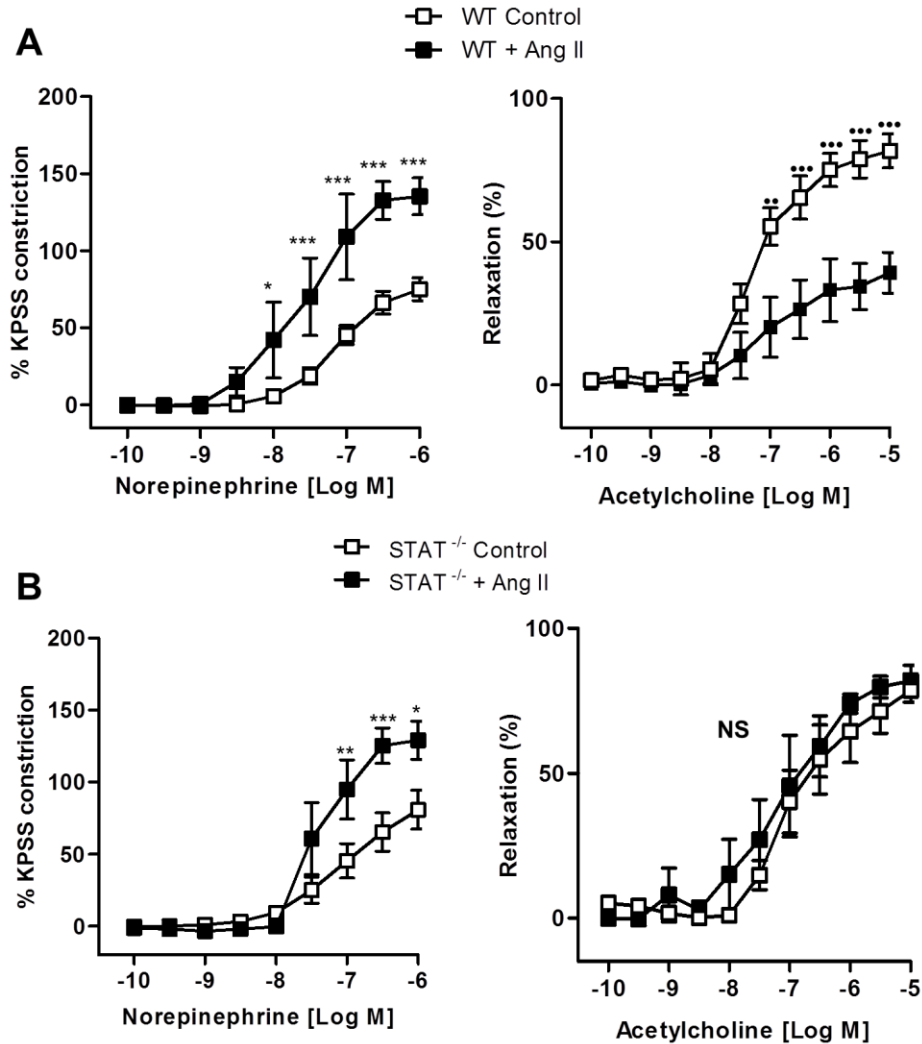
**Fig. 4-3. Inflammatory cell infiltration in heart of animals exposed to Ang II.** **A**, Representative images of CD45 immunohistochemical staining of heart tissue. **B**, The column graph represents the number of positive cells per tissue slide examined (data are presented as mean  $\pm$  SEM,  $n \geq 4$  mice/group).



**Fig. 4-4. STAT1 deficiency reduces heart fibrosis.** **A**, Representative images of Sirius red staining of the heart tissue. **B**, RNA from heart was isolated and subjected to qRT-PCR for collagen (*col3a1*) using *GAPDH* as internal control. **B**, additionally, to evaluate cardiac collagen content, tissue hydroxyproline assay was performed (**C**). Data for  $n \geq 4$  per group  $\pm$  SEM.



**Fig. 4-5. STAT1 deficiency reduced expression of proinflammatory mediators in animals exposed to Ang II.** RNA from heart was isolated and subjected to qRT-PCR for *Cxcl10*, *MCP-1 (CCL2)*, *Cxcl9* and *iNOS* using *GAPDH* as internal control  $n \geq 4$  per group  $\pm$ SEM.

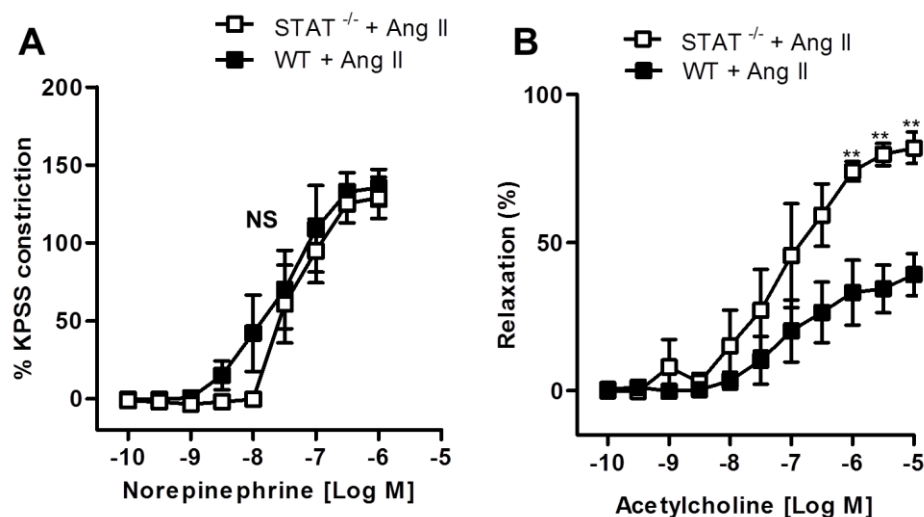


**Fig. 4-6. Macrovascular function in WT and STAT1-deficient mice exposed to Ang II vs. control.** WT and STAT1<sup>-/-</sup> mice were treated for 14d with 1,5μg/g/day Ang II, administered subcutaneously via osmotic minipump. Isolated aortic rings were tested for response to norepinephrine and acetylcholine using the wire myograph. Noradrenaline was used in stepwise increased, cumulative concentration ranging from 10<sup>-11</sup> to 10<sup>-6</sup> mol/L and followed by acetylcholine dose-response curve from 10<sup>-10</sup> to 10<sup>-5</sup> mol/L **A**, Reaction to noradrenaline and acetylcholine in WT. \*\*\*p0.001< vs. WT control; \*p<0.001 vs. WT control; ••p0.001< vs. WT control; ••p0.01< vs. WT control. **B**, Reaction to noradrenaline and acetylcholine in aortic rings isolated from STAT1<sup>-/-</sup>. \*\*\*p0.001< vs. STAT1<sup>-/-</sup> control; \*p0.05< vs. STAT1<sup>-/-</sup> control; NS not significant. Aortas isolated from at least 3 animals per group were taken ±SEM. Two-way ANOVA test with Bonferroni post hoc test was used.



### Stat1-mediated effects of Ang II-infusion on vessel wall

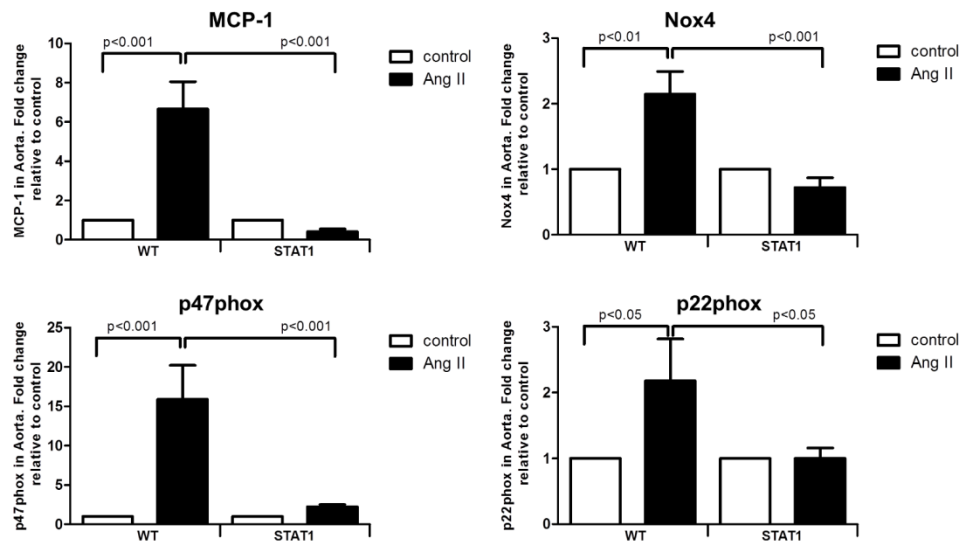
One key factor for Ang II-induced hypertension is the macrocirculatory function. Using myograph device, the noradrenaline dependent vasoconstriction and acetylcholine dependent relaxation was assessed. Aortic rings from WT mice exposed to Ang II had increased vasoconstriction responses to noradrenaline, presented as a percentage of maximal constriction to KPSS. (Fig. 4-6 A left panel) (Control WT: 75% vs. Ang II WT: 135%). In line with these results we observed that chronic Ang II infusion impaired endothelium-dependent vasodilatation (Fig. 4-6 A right panel) (control WT: 81.7% vs. Ang II WT: 39.2%). Aortic rings from STAT1<sup>-/-</sup> mice exposed to Ang II had increased vasoconstriction responses to noradrenaline, (Fig. 4-6 B left panel) (control STAT: 80.8% vs. Ang II STAT1<sup>-/-</sup>: 128.9%). Interestingly, STAT1 animals infused with Ang II did not reveal impaired endothelium-dependent vasodilatation (Fig. 4-6 B right panel) (control STAT1: 81.9% vs. Ang II STAT1<sup>-/-</sup>: 78.6%).



**Fig. 4-7. STAT1 protects against endothelial dysfunction.** Macrovascular function in WT mice exposed to Ang II vs STAT1<sup>-/-</sup> was analyzed via wire myograph. Noradrenaline was used in stepwise increased, cumulative concentration ranging from 10<sup>-11</sup> to 10<sup>-6</sup> mol/L (A) and followed by acetylcholine dose-response curve from 10<sup>-10</sup> to 10<sup>-5</sup> mol/L (B). \*\*p<0.01, NS not significant. Data for n≥3 per group ±SEM. Two-way ANOVA test with Bonferroni post hoc test was used.

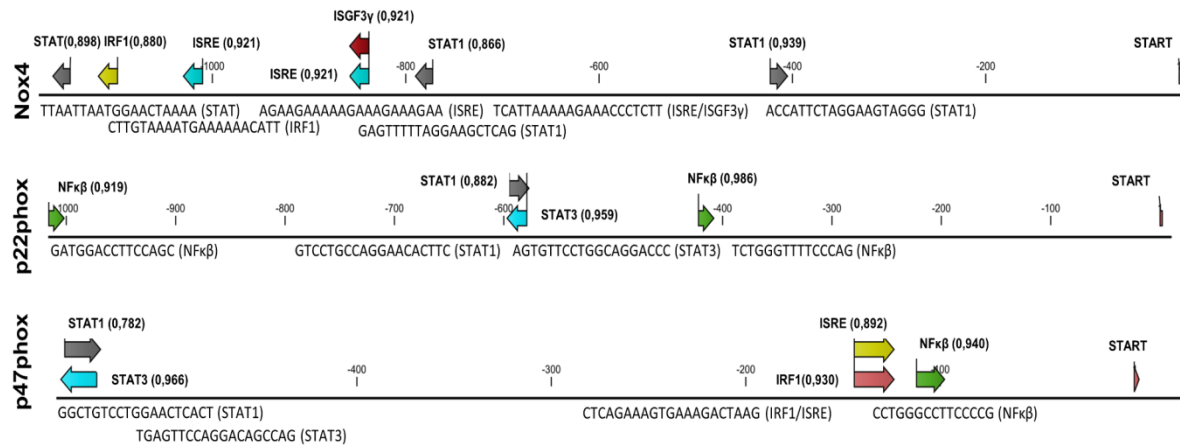
In the next step we compared aortic function of WT and STAT1<sup>-/-</sup> animals exposed to Ang II. STAT1<sup>-/-</sup> and WT groups had a similar maximum vasoconstriction after chronic angiotensin infusion (Fig. 4-7 A). Most importantly, only WT tend to have ameliorated acetylcholine

dependent vasodilation in aortas which indicates that STAT1<sup>-/-</sup> are protected from Ang II infused vascular dysfunction (Fig. 4-7 B).



**Fig. 4-8. STAT1 deficiency reduces expression of ROS marker in animals exposed to Ang II.** RNA from aorta was isolated and subjected to qRT-PCR for *Ccl2* (MCP-1), *Nox4*, *p47phox*, *p22phox* using *GAPDH* as internal control n≥4 per group ±SEM.

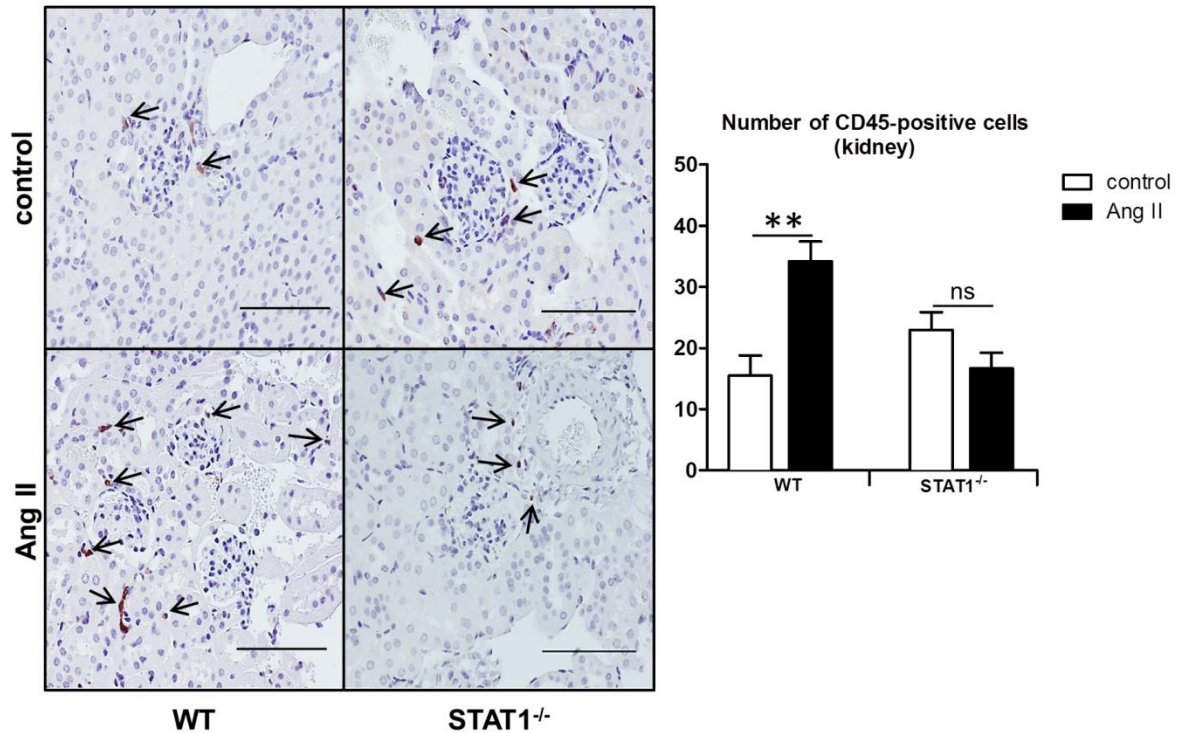
Similarly as in the case of the heart, increased expression of inflammatory marker was detected in the aortic tissue of WT but not STAT1<sup>-/-</sup>. WT infused with Ang II revealed increased abundance of *Mcp-1* (*Ccl2*) as compared to the control (Fig. 4- 8, upper left panel). To identify the potential cause of the abolished acetylcholine-mediated vessel relaxation, we verified presence of oxidative burst. ROS are known to be upregulated during Ang II stimulation and are associated with endothelial dysfunction. Indeed, we observed increased expression of oxidative stress markers *p22phox*, *p47phox* and *Nox4* only in aortas of WT but not STAT1<sup>-/-</sup> exposed to Ang II (Fig. 4-8). Promoter analysis of *p22phox*, *p47phox* and *Nox4* revealed presence of binding sites characteristic for STAT1, STAT3 but also for IRF1, NFκB and ISGF3γ complex, providing additional evidence for the direct role of STAT1 in regulating NADPH oxidase expression (Fig. 4-9).



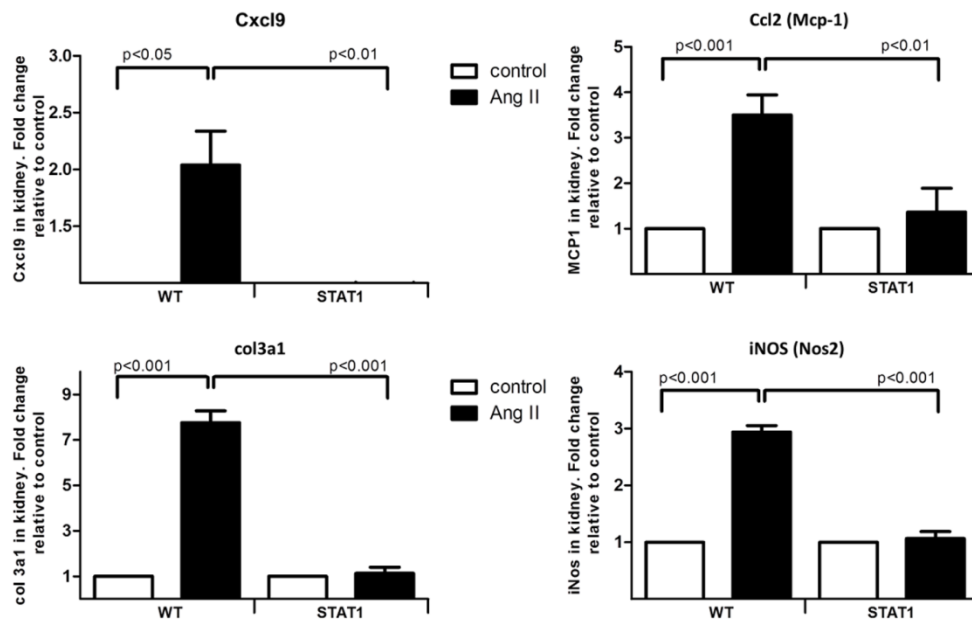
**Fig. 4-9. Promoter analysis of *Nox4*, *p47phox*, *p22phox*.** The promoter regions from -1000 to +100bp were searched for binding sites using a prediction algorithm (MatInspector, GENOMATIX software, <http://www.genomatix.de/> [160]). To locate binding sites for STAT1, STAT3 IRF1 predefined matrices were used (V\$IRF1.01 V\$ISGF3G.01 V\$ISRE.01 V\$ISRE.02V\$CREL.01 V\$NFKAPPAB.01 V\$NFKAPPAB.02 V\$NFKAPPAB65.01 V\$STAT.01 V\$STAT1.01 V\$STAT1.02, V\$STAT3.02 V\$STAT3.01) Only sites with core similarity above 0.85 were selected. Start indicates position of ATG codon. In brackets matrix similarity score is given (the higher the number, the more conserved sequence is present).

#### Stat1-mediated effects of Ang II-infusion on kidney.

Next, we evaluated role of STAT1 in Ang II mediated renal damage. Alike in heart we could observe upregulation of inflammatory mediators *Cxcl9*, *Ccl2* as well as fibrotic (*col3a1*) and nitric oxide marker (*Nos2*) in WT but not STAT1<sup>-/-</sup> mice upon chronic infusion of Ang II (Fig. 4-11). This was accompanied by reduced infiltration of CD45<sup>+</sup> cells STAT1<sup>-/-</sup> animals (Fig. 4-10).

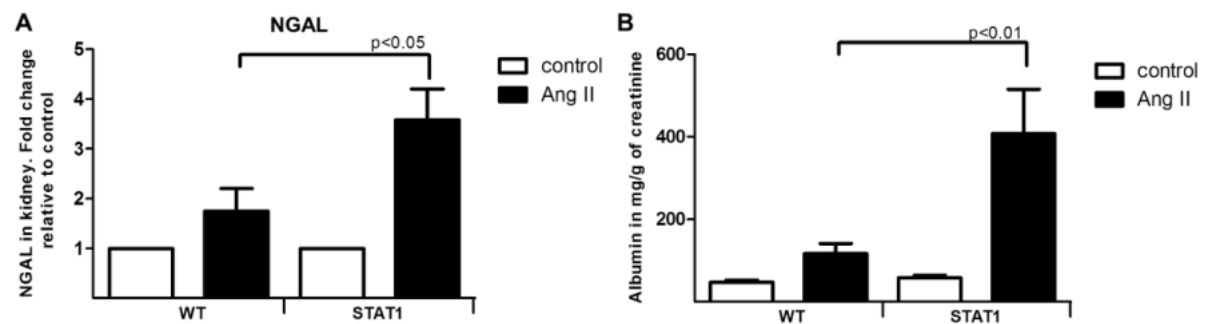


**Fig. 4-10. Inflammatory cell infiltration in kidney of animals exposed to Ang II.** A, Representative images of CD45 immunohistochemical staining of kidney tissue. B, The column graph represents the number of positive cells per tissue slide examined (data are presented as mean  $\pm$  SEM,  $n \geq 4$  mice/group).

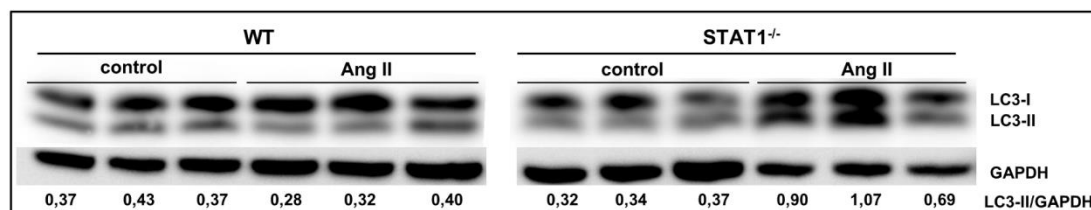


**Fig. 4-11. Reduced expression of proinflammatory mediators in kidneys of STAT1-deficient animals exposed to Ang II.** RNA from kidney was isolated and subjected to qRT-PCR for *Cxcl9*, *MCP-1* (*Ccl2*), *col3a1* and *iNOS* using *GAPDH* as internal control  $n \geq 4$  per group  $\pm$  SEM.

Interestingly, despite of decreased inflammatory cells infiltration, STAT1<sup>-/-</sup> exposed to Ang II revealed markedly higher expression of Neutrophil gelatinase-associated lipocalin (NGAL) as well as massive increase in albuminuria, indicating increased tubular and glomerular damage in the absence of STAT1 (Fig. 4-12).



**Fig. 4-12. STAT1 deficiency disturb kidney function.** **A**, RNA from kidney was isolated and subjected to qRT-PCR for *NGAL* using *GAPDH* as internal control. **B**, Urine was collected in metabolic cage for 24h. Albumin and creatinine was measured using commercially available ELISA.  $n \geq 4$  per group  $\pm$ SEM.



**Fig. 4-13. STAT1 participates in autophagy.** Protein extracts from kidneys of 3 animals exposed to Ang II and their controls (3) were analysed by Western blot for LC3.

We hypothesized disturbed autophagy to be a cause of ameliorated kidney damage. Thus, we verified expression of one of the most important marker of autophagy, LC3-II protein which is created by cleavage of soluble LC3-I [236]. While expression of LC3-II did not change in WT animals treated with Ang II, expression of LC3-II was increased in STAT1-deficient mice exposed to Ang II (Fig. 4-13). These results indicate that STAT1 not only activates expression of proinflammatory genes that are important for immune cell infiltration but also may disturb processes related to autophagy and thus influence organ damage.

## Discussion

Although the mechanism is not fully understood, several studies indicate that Ang II not only upregulates blood pressure but also affects the immune response by amplifying genes related to inflammation. The results presented in this chapter indicate that STAT1 may participate in an Ang II-mediated inflammatory response. We have shown that chronic Ang II infusion causes similar increases in systolic BP and heart hypertrophy in WT- and STAT1-deficient mice. However, STAT1<sup>-/-</sup> animals with Ang II-induced hypertension exhibited highly ameliorated expression of proinflammatory mediators (*Cxcl9*, *Cxcl10*, *Ccl2*, *iNOS*) in the heart and kidney, which was correlated with reduced CD45<sup>+</sup> cell infiltration, decreased production of extracellular matrix components and, consequently, reduced organ injury. Recent evidence pointed to the importance of the JAK/STAT pathway in Ang II-mediated hypertension and hypertensive end-organ damage. Expression of IFN $\gamma$  was upregulated in an Ang II-induced model of hypertension [31], and IFN $\gamma$ -deficient mice had reduced heart infiltration by macrophages, which was associated with decreased fibrosis [73, 74].

Furthermore, these experiments indicated that, at least partially, the function of Ang II is mediated through the activation of lymphocytes and subsequent IFN $\gamma$  secretion [73, 74, 221]. Importantly, recent studies pointed to the fact that also Ang II can reveal its actions *via* activation of important components of the JAK/STAT pathway [223]. Ang II cooperates with the JAK2 kinase to induce vessel contraction, and inhibition of JAK2 phosphorylation blocks blood pressure elevation [224, 225]. Our results demonstrate that the mechanism of Ang II blood pressure regulation is STAT1-independent. STAT1 infused with Ang II had similar increases in systolic BP and heart hypertrophy as WT animals (Fig. 4-2). However, STAT1 animals exposed to Ang II revealed reduced expression of inflammatory markers and reduced inflammatory cell infiltration (Figs. 4-3-4-5). Several studies have indicated the contribution of inflammation to the progression of fibrosis, which is defined by the accumulation of an extracellular matrix component (e.g. collagen, fibronectin) and gradual loss of organ function [237, 238]. During chronic Ang II infusion, resident and infiltrating leukocytes promote injury by production of proinflammatory cytokines and ROS as well as activating myofibroblasts that express extracellular matrix components. Thus, expression of chemokines is one of the initial steps of organ damage and preventing their recruitment may ameliorate tissue injury. Indeed,

literature data support this theory. Mice lacking the receptor for Ccl2 (Mcp1) infused with Ang II had ameliorated vascular inflammation and remodeling accompanied by reduced ROS production and fibrosis as compared to the CCR2<sup>+/+</sup> animals [239, 240]. Expression of Mcp1 correlated with macrophage infiltration and albuminuria in patients with chronic kidney disease [241]. Elevated levels of chemokines were observed in patients with hypertension [211]. Increased levels of Cxcl10 and Ccl2 were found in patients with essential hypertension, and treatment with angiotensin-converting-enzyme inhibitor lowered their expression [242, 243]. Our data indicate that STAT1 plays a crucial role in regulating chemokine expression in response to Ang II stimulation. Mice lacking STAT1 had reduced expression of Ccl2 and Cxcl10 but also Cxcl9 as compared to WT infused with Ang II (Fig. 4-5). These results, together with reduced production of ROS, may explain ameliorated fibrosis in STAT1<sup>-/-</sup> animals.

To answer the question whether inflammatory activity of STAT1 is directly mediated through the action of Ang II, we performed *in vitro* stimulation of vascular cells with Ang II (data not shown). Although we were able to observe temporal STAT1 tyrosine phosphorylation, we could not detect any of the proinflammatory markers that were observed in our *in vivo* model. This suggests that action of Ang II on the activity of STAT1 is indirect and very likely IFN $\gamma$ -dependent. Other potential activators of STAT1 may be related to the TLR4 and NF $\kappa$ B pathway [244, 245]. Spontaneously hypertensive rats demonstrated enhanced expression of the TLR4 receptor in cardiac tissue and the central blockade of TLR4, improved cardiac function and attenuated myocardial inflammation [101, 246]. Moreover, NF $\kappa$ B suppression markedly attenuated Ang II-induced organ injury [247]. It is very likely that functional cooperation between NF $\kappa$ B and STAT1 also appears in such a model of inflammation and is essential for efficient expression of many chemokines, such as Cxcl9, Cxcl10, or Ccl2.

We found increased production of chemokines in the vasculature which was accompanied by increased expression of oxidative stress markers in WT but not STAT1<sup>-/-</sup> exposed to Ang II (Fig. 4-8). Accelerated production of ROS by NADPH oxidases initiates endothelial dysfunction, a hallmark of the onset and progression of vascular disease [248]. There are seven members of the family, of which Nox1, Nox2, Nox4 and Nox5 enzymes are expressed in cardiovascular tissues. These enzymes, together with their regulatory subunits, e.g. p22phox or p47phox,

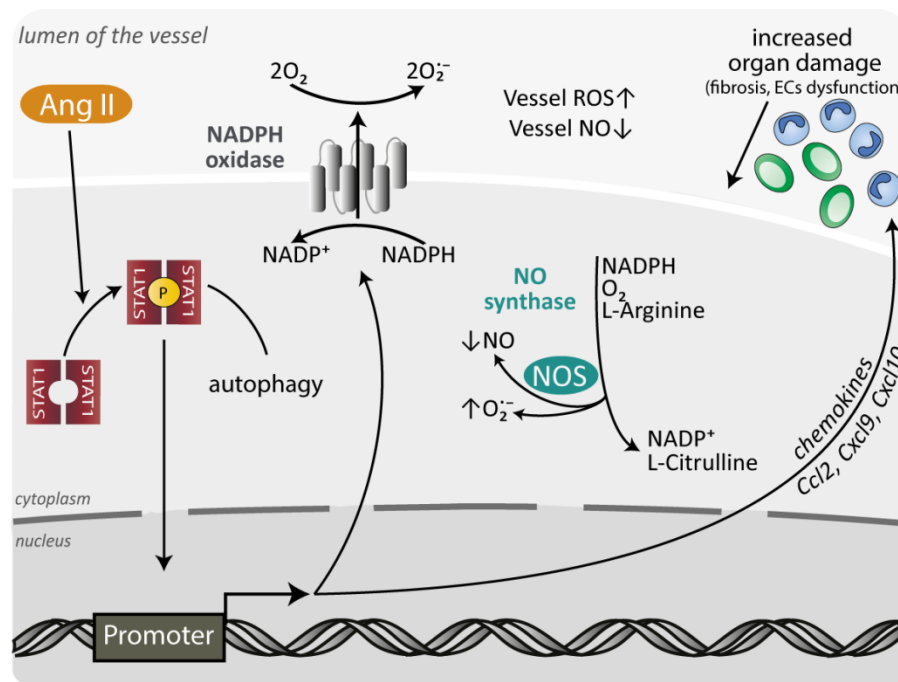
in pathological conditions contribute to progression of the disease, including hypertension [249]. Interestingly, there is cooperation between the JAK/STAT1 pathway and NADPH oxidases. Some studies have indicated that activation of the JAK/STAT pathway by Ang II depends on the Nox-derived ROS [227]. Recently, expression of this regulatory component of NADPH oxidase was hypothesized to be STAT3, but also STAT1-dependent [226]. Our *in silico* promoter analysis (Fig. 4-9) confirmed the observation indicating that action of STAT1 is not only limited to the regulation of chemokine expression but may also affect expression of NADPH oxidases in the vessel wall. In addition to the STAT3-binding sequence, we could identify the presence of a characteristic sequence for the STAT1, IRF1, NFκB and ISGF3γ complex, thus indicating possible functional cooperation of these transcription factors in regulating NADPH oxidase expression.

Increased production of ROS by NADPH oxidases causes reduced nitric oxide (NO) bioavailability leading to ameliorated vessel relaxation [250] (Fig.4-6, 4-7). Dilation of the vessel is mediated mainly through the conversion of L-arginine to L-citrulline and nitric oxide synthesis by NO synthase with the involvement of many cofactors, including NADPH, FMN, FAD, calmodulin, heme, and tetrahydrobiopterin (BH4). However, in the presence of ROS, NO synthase is “uncoupled”. At that stage the enzyme is not able to convert amino acids but is still able to transfer electrons from NADPH to molecular oxygen and to form superoxide ( $O_2^-$ ) [251]. This process reduces the amount of available NO and promotes endothelial dysfunction. Due to lower NO bioavailability, vessels are not able to fully dilate in response to the release of endothelial-mediated vasodilators such as acetylcholine. Thus, increased expression of ROS has an effect on macro- and microcirculatory function. Treatment with Ang II led to increased maximal response and the left shift in noradrenaline sensitivity in WT animals. Furthermore, there was a highly reduced response of endothelial cells to acetylcholine. Compared to WT, STAT1 animals treated with Ang II had similar maximal constriction. As there was no difference in systolic BP, this result was expected. However, in contrast to WT, STAT1<sup>-/-</sup> exhibited preserved endothelium function manifested by unaffected response to acetylcholine. Ameliorated ROS production together with decreased expression of proinflammatory mediators may explain the protective phenotype of STAT1<sup>-/-</sup> animals in the vasculature.



Similarly as with the heart and vasculature, abolished expression of inflammatory genes and markers of fibrosis in the kidneys of STAT1-deficient animals was detected (Figs. 4-10, 4-11). Nonetheless, we observed increased expression of NGAL, a marker of tubulointerstitial damage, and highly increased albuminuria indicating enhanced Ang II-induced glomerular damage in STAT1-deficient mice (Fig. 4-12). The mechanism which explains this phenomenon may be related to autophagy [252]. Autophagy is a prosurvival, highly regulated catabolic process responsible for the degradation of cytoplasmic components. It is based on the formation of double membrane vesicles containing damaged proteins or organelles which fuse with the lysosome, thus leading to digestion of their content. This process is essential for cells exposed to stress factors such as hypoxia, infection, or oxidative stress, and alteration of autophagy may be a source of a pathological state [253, 254]. Autophagy can also regulate inflammation and fibrosis [255, 256]. Zhao et al. showed that an autophagy-deficient mouse exposed to Ang II had an increased level of reactive oxygen species (ROS) production as well as increased levels of inflammation and cardiac injury. Ang II was found to promote autophagy in podocytes and its abrogation triggered glomerulopathy and proteinuria [257, 258].

Our results indicate that a lack of STAT1 disturbed autophagy in the kidney (Fig. 4-13). STAT1 animals exposed to Ang II had higher levels of LC3-II as compared to WT animals. These results are in line with studies performed by Marko et al. [74]. IFN $\gamma$  receptor knockout mice infused with Ang II had highly elevated levels of albuminuria accompanied by a decreased number of podocytes and an increased amount of LC3 in the glomeruli. LC3 is crucial for vesicle (autophagosome) formation and maturation [236]. After synthesis, proLC3 is processed to LC3-I and conjugated with phosphatidylethanolamine to form LC3-II. Increased levels of LC3-II may indicate either enhanced autophagosome synthesis (increased autophagy) or reduced vesicle turnover (decreased autophagy). In order to interpret observable changes in the LC3 amount, further experiments including transmission electron microscopy (TEM) as well as stimulation with compounds inhibiting autophagosome degradation are needed [259].



**Fig. 4-14. Hypothesized role of STAT in Ang II-mediated end-organ damage.** Activity of STAT1 is crucial for efficient expression of *Cxcl9*, *Cxcl10* or *Ccl2* upon Ang II infusion. Enhanced chemokine expression facilitates leukocyte trafficking and tissue injury. Additionally, STAT1 affects expression of iNOS and important components of NADPH oxidase, thus further contributing to ROS production. For details see text.

Moreover, it is still unclear how STAT1 affects autophagy, and studies that have been performed until now are partially contradictory. Increased activation of STAT1 caused by histone deacetylase 4 was associated with ameliorated autophagy, accelerated inflammation and podocyte injury in the model of diabetic nephropathy [260]. McCormick et al. showed that STAT1<sup>-/-</sup> mice undergoing ischemia-reperfusion (I/R) injury had smaller infarcts and enhanced levels of autophagy. Inhibition of autophagy abrogated cardioprotection observed in STAT1<sup>-/-</sup> animals following I/R injury [232]. These results indicate that STAT is involved in the inhibition of autophagy. STAT1 was also found to co-immunoprecipitate with LC3, and hearts from STAT1<sup>-/-</sup> subjected to *ex vivo* I/R had an increased number of damaged mitochondria located within double-membrane structures. The authors suggested an increased rate of autophagy in the absence of STAT1 [261]. In line with these results, STAT1-deficient human fibrosarcoma cells exhibited increased autophagic activity [262].

In contrast, another group proposed the role of STAT1 in the induction of autophagy. Formation of the autophagosome in the breast cancer cell line was STAT1-dependent [263]

and IFN $\gamma$  was found to induce cell autophagy [264, 265]. Taken together, these data imply a novel STAT1-dependent and tissue-specific role in the regulation of autophagy.

Altogether, the results presented in this chapter provide experimental evidence for the function of STAT1 in Ang II-mediated tissue injury (Fig. 4-14). During Ang II infusion, activated STAT1 promotes expression of Ccl2, Cxcl10, Cxcl9 chemokines, thus leading to increased CD45<sup>+</sup> infiltration. Activated leukocytes induce the oxidative burst, thus promoting damage and contributing to tissue fibrosis and organ malfunction. Additionally, the transcriptional activity of STAT1 in the vessel wall affects expression of iNOS and important components of NADPH oxidase, thus further contributing to ROS production. An increased oxidative burst limits the amount of bioavailable NO and causes endothelial dysfunction. In contrast to the vasculature lack of STAT1 in the kidney resulted in deterioration of the organ function. This phenotype occurred most likely due to the impaired autophagy mechanism after stimulation with Ang II.

## **Chapter 5**

### **STAT1 and IRFs in Cardiovascular disease**

Cardiovascular diseases are globally the leading cause of death. According to the World Health Organization, the number of people that will die from CVDs will increase to 23.3 million in 2030 [1, 2]. In Poland, cardiovascular disorders are the reason for 46% of total deaths [266], and atherosclerosis accounts for 18% of those deaths. Therefore, a detailed understanding of the mechanisms contributing to the progression of this type of diseases together with prevention is a true challenge to the modern health care system. Recently, more attention has been paid to the role of the immune component in the progression of CVDs. Transcription factor STAT1 together with downstream-activated IRFs play a crucial role in regulating the immune response. In this chapter we further summarize the findings presented in the thesis and discuss potential applications as well as future research directions.

#### **STAT1-dependent signal integration between IFN $\gamma$ and TLR4 in non-immune cells**

First, we studied the role of STAT1 and IRFs as potential regulators of inflammation in non-immune cells. We hypothesized that STAT1- and IRF-mediated gene expression accelerates the inflammatory response which negatively affects the cardiovascular system. Indeed, the results presented in Chapter 2 and further extended in Chapter 3 showed that in addition to myeloid and lymphoid cells, STAT1 in the vascular cells together with downstream-activated IRF1 and IRF8 orchestrate a platform for crosstalk between the JAK/STAT and TLR4 pathway. In addition, we were able to, for the first time, identify IRF8 in cells from the vasculature. Interestingly, by analyzing expression profiles in non-immune cells we could distinguish cell type-specific regulatory mechanisms activating the IFN $\gamma$ - and LPS-mediated response. In cells from the vasculature, synergistic amplification of the genes was dependent on an increased amount of phosphorylated STAT1 and its subsequent interaction with LPS-activated transcription factors. In contrast to the ECs and VSMCs, we did not detect increased phosphorylation of STAT1 in stimulated proximal tubular cells. We postulated the mechanism in which not increased STAT1 activity but increased expression of STAT1-mediated transcription factors and subsequent interaction with NF $\kappa$ B facilitated signal integration of the

downstream genes. Indeed, expression of IRF1 was highly amplified upon treatment with IFN $\gamma$ , but not upon LPS. These results indicated the involvement of IRF1 in the synergistic amplification of downstream genes in proximal tubular cells and emphasized tissue-specific mechanisms regulating signal integration.

Although not fully understood, functional cooperation between STAT1 and IRF8 was observed in immune cells. IRF8 abundance was synergistically amplified upon treatment with IFN $\gamma$  and LPS in macrophages [115]. Moreover, its expression and subsequent interaction with IRF1 was mandatory for *Nos2* activation. IRF8-deficient macrophages stimulated with IFN $\gamma$  did not produce nitrite [208, 267]. Our results pointed to STAT1-dependent expression and synergistic amplification of IRF8 in VSMCs and ECs. However, the precise role of IRF8 still has to be addressed. First, it is not known which genes are regulated by IRF8 in non-immune cells. Based on evidence in the literature, we studied the expression of *Nos2* and *Ccl5*, although it is very likely that there are other IRF8-dependent genes. Microarray experiments combined with chromatin immunoprecipitation (ChIP) sequencing or ChIP-PCR performed on IRF8-deficient ECs and VSMCs should answer this question. Moreover, the precise mechanism by which IRF8 contributes to gene expression upon signal integration is still not fully understood. Zhao et al. suggested that IRF8 promotes crosstalk between TLR and IFN $\gamma$  signaling through interaction with crucial components of the TLR4 pathway [115]. Additionally, IRF8 was found to interact with other members of the TLR family such as TLR3 and TLR2, which have recently been recognized as playing a role in the cardiovascular system [268]. It would be interesting to verify whether a similar phenomenon occurs in the vascular cells and whether this mechanism is related to the activity of STAT1. Recently, IRF8 was proposed as playing a crucial role in regulating the induction of the M1 phenotype in macrophages [195]. These classically activated macrophages express a high level of pro-inflammatory cytokines and contribute to the progression of cardiovascular disease. Additionally, modification of IRF8 by small ubiquitin-like modifiers (SUMO) inhibits IRF8 action and, as a consequence, the macrophage phenotype switch that prevents expression of proinflammatory mediators such as IL12p40 [269]. Since expression of IRF8 in ECs and VSMCs seems to be STAT1-dependent, it is tempting to suggest a mechanism where IRF8 may in part account for the “immune cell-specific” STAT1-dependent functions of IFN $\gamma$ . In this process the ECs and VSMCs change their phenotype and actively participate in amplifying and sustaining the inflammatory process. As such, IRF8 can

be considered as an interesting therapeutic target that modulates the STAT1-mediated proinflammatory response.

### **STAT1 and IRFs in atherosclerosis and hypertension**

The results presented in Chapters 1 and 2 strongly suggest that STAT1 together with upregulated IRF1, IRF8 and the activated TLR4 pathway coordinate a platform for synergistic amplification of genes, which results in phenotypic changes of the vascular cells and leads to amplified pro-atherogenic responses. Thus, ECs and VSMCs can be considered not only as passive receivers of the immune-driven stimuli but also as active modulators of vessel damage. Increased activation of STAT1 and STAT1-dependent IRF1 and IRF8 (e.g. in the presence of JAK/STAT and TLR4 agonists) can be the reason for synergistic amplification of multiple chemokines, adhesion molecules and antiviral and antibacterial response proteins which, in turn, facilitate white blood cell trafficking and further contributes to the progression of cardiovascular disease such as atherosclerosis. Microarray analysis performed on stimulated VSMCs identified a whole set of STAT1-dependent genes that were prone to synergistic amplification. Promoter analysis predicted the presence of transcription binding sites containing GAS, ISRE or NFkB elements either alone or in different combinations. Moreover, immunohistochemistry performed on human specimens revealed the presence of phosphorylated STAT1 as well as STAT1-dependent genes in carotid plaque.

Atherosclerosis is not the only immune-driven CVD disease. Recent data indicate that also in hypertension the immune system is an important contributor to its progression. In Chapter 4 we studied the role of STAT1 in an Ang II-induced model of hypertension and tissue injury. Just as in the model of atherosclerosis, here too we could identify several STAT1 downstream genes that were upregulated upon treatment with Ang II, including genes involved in leukocyte trafficking and oxidative burst. Immunohistochemistry together with the analysis of vessel function confirmed the importance of STAT1 in regulating Ang II-mediated tissue damage. Based on our results we hypothesized that Ang II-increased leukocyte infiltration is at least partially mediated through the transcriptional activity of STAT1. Increased expression of chemokines resulted in increased CD45<sup>+</sup> cell infiltration, accelerated oxidative burst and,

as such, contributed to tissue fibrosis and organ malfunction. Importantly, our *in silico* analysis predicted the presence of STAT- binding sites in the promoter region of genes involved in the regulation of oxidative stress. These results allow us to suggest that the action of STAT1 is not only limited to the induction of chemokine synthesis but can also actively participate in promoting tissue injury by stimulation of NADPH oxidase expression. Thus, due to its involvement in the regulation of ROS production, STAT1 might be considered as an interesting therapeutic target. This is particularly important in the context of CVDs, as ROS has been associated in the pathogenesis of many of them. Indeed, some studies have pointed to the role of the STAT family in regulating NADPH expression [226]. Johnson et al. showed that the inhibitors of STAT3 prevented Ang II-mediated oxidative stress and EC dysfunction [270]. Since all known inhibitors of the JAK/STAT pathway that block STAT3 also interfere with STAT1, these results suggest the involvement of STAT1 and STAT3 in the regulation of oxidative stress. Our *in vivo* studies together with promoter analysis support this hypothesis and open up a new and interesting research area.

Interestingly, abolished expression of inflammatory genes and markers of fibrosis in the kidneys of STAT1-deficient animals did not improve organ function but surprisingly accelerated the injury. We observed increased albuminuria indicating enhanced Ang II-induced glomerular damage in STAT1-deficient mice. Our findings indicate that systemic inhibition of factors that participate in inflammation in certain conditions may not be beneficial to organ function. Moreover, we proposed that disturbed autophagy was the reason for ameliorated organ damage in the absence of STAT1. However, how exactly STAT1 modulates autophagy remains an open question. Further experiments with the use of tissue-specific knockouts are mandatory to determine the role of STAT1 in this aspect.

Similar to atherosclerosis, the role of the JAK/STAT pathway in obesity and obesity-related insulin resistance has been investigated. This is particularly important as obesity is associated with increased cardiovascular risk [271-273]. Compared with the control, animals fed a high-fat diet had an increased amount of infiltrating Th1 cells and produced a higher amount of IFN $\gamma$ . Obese IFN $\gamma$ -deficient mice expressed less proinflammatory mediators such as Ccl2 or TNF $\alpha$  and had better glucose tolerance [274]. McGillicuddy et al. demonstrated that stimulation of adipocytes with IFN $\gamma$  induces insulin resistance and ameliorates triglyceride

storage [275]. The authors suggested that JAK1 and STAT1 are crucial players of these events. Indeed, our preliminary studies suggested the role of STAT1 in the progression of insulin resistance. Genome-wide studies comparing the expression profiles of fat pads isolated from WT and STAT1<sup>-/-</sup> animals fed a high-fat diet revealed significant changes in the expression of genes involved in the glucose metabolism (data not shown). Nevertheless, it is not known how STAT1 modulates adipocyte functions and whether STAT1-mediated alterations contribute to vessel function. These issues will be the subject of further investigation.

Besides STAT1, recent data indicate the involvement of proteins from the IRF family in the progression of cardiovascular disease. IRFs were found to be involved in the regulation of cardiac hypertrophy and remodeling in response to stress [276]. Expression of IRF7 was downregulated upon treatment with Ang II or phenylephrine in cardiomyocytes. In line with these results, *in vivo* studies performed on animals with disturbed IRF7 expression revealed the crucial role of IRF7 in the regulation of cardiac hypertrophy [277]. In this model, aortic constriction was performed and cardiac hypertrophy together with heart failure were investigated. Similarly to IRF7, IRF3 and IRF9 were protective against pressure overload-induced hypertrophy. Interestingly, IRF8 has, on the one hand, been found to enhance smooth muscle cell proliferation and neointima formation but, on the other hand, IRF8 has also been evidenced to protect against cardiac hypertrophy and heart failure in a model of pressure overload [88, 278]. Expression of IRF1 was found to be altered in the tissue samples of patients with heart disease and in mice subjected to a model of pressure overload [279]. Mice overexpressing IRF1 had increased ventricular dilation and fibrosis and dysfunction. Jiang et al. suggested that IRF1 participates in heart damage by direct activation of iNOS in response to stress conditions, thus further supporting the role of iNOS [279, 280]. Altogether, new evidence indicates the novel role of IRFs in the development of cardiovascular diseases. Yet their function is ambiguous; for example, it is still not known how IRFs are activated and whether their activation is interferon- and STAT-mediated. Therefore, further studies using different animal models are needed to determine the function of IRFs in CVDs.



### **STAT1 and IRFs as therapeutic targets**

The data presented here but also data published by other groups suggest that STAT and IRF proteins can be considered as therapeutic targets affecting inflammatory processes during CVDs. Until now there have been no admitted drugs that specifically target STAT1 or IRF8. Besides STAT3, research on potential inhibitors of other STATs including STAT1 is very limited [281]. To the best of our knowledge there is no information on the modulators of IRF action.

Several inhibition approaches that interfere with proteins from the STAT family have been suggested [281]. Among them we can distinguish strategies based on indirectly blocking STAT action, such as antibody-mediated prevention of pathway activation or inhibition of JAK kinases phosphorylation. Other strategies interfere with the binding of active STAT complexes to the promoters by applying decoy oligodeoxynucleotides, or they interfere with STAT mRNA using antisense oligonucleotides. Finally, the most common approach includes blocking the STAT SH2 domain and subsequent prevention of phosphorylation and dimer formation. Unfortunately, there are some pitfalls for such strategies. Targeting proteins upstream from STAT will result in interference of molecules that are not necessarily related to one pathway; for example, inhibition of JAK2 kinase phosphorylation with a compound such as AG490 will result in suppression of not only STAT1 but also STAT3. Inhibition of several targets at once may contribute to increased toxicity.

Moreover, crystal structures are available only for a few members of the family, and as such quality models for virtual screening are poor [282]. STAT proteins share important structural similarities, and without detailed crystallography of human STAT (or their homologs), designing a specific STAT compound will be difficult. Indeed, our results showed that the inhibitors which were considered as specific toward STAT3 could also affect the activity of STAT1 and *vice versa*.

Recently, our group presented a new strategy for the screening and validation of pre-selected STAT inhibitors [282]. Based on the available crystal models, we generated 3D structures for all human STATs. By using these models we could verify whether pre-selected compounds are specific to the targeted STAT or can associate with other proteins of the family. Following *in silico* comparative screening, we suggested cell-based multiple STAT activation *in vitro*

phosphorylation assay. This assay allows to verify the effect of the inhibitory compounds on the activity of STAT1 (constitutive or ligand-induced).

Although targeting of STAT1 is an interesting research area, we might expect potential side effects due to the extensive regulatory features of STAT1. Fludarabine, the commercially available drug that is known to inhibit STAT1 action, is toxic [283]. Naturally, this could be explained by the reduced specificity of fludarabine to STAT1, but there is also other evidence indicating the potential risk of using STAT1 inhibitors. In the model of Ang II-induced abdominal aortic aneurysm, IFN $\gamma$ -deficient mice had increased aneurysm incidence and death, although there was decreased atherosclerotic plaque formation [284]. In line with these results, Eagleton et al. noticed that loss of STAT1 was associated with higher incidence of aortic rupture [285]. In contrast, others noticed that mutation resulting in a gain of STAT1 phosphorylation manifested aneurysm incidence [286]. Together with our results concerning kidney function as presented in Chapter 4, these studies postulate careful consideration in using the potential inhibitors of STAT1 and enforce a more specific approach. Importantly, our experiments suggest not only STAT1 but also IRFs as potential targets of novel drugs. Indeed, recent data presented in the subsection above seem to support the relevance of such an approach. Considering the structural similarities among proteins from the IRF family, we believe that the strategy proposed for STAT proteins can also be applied for IRFs.

### **Diagnostic potential of STAT1 and IRFs**

A biomarker is defined as “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention” [287]. Biomarkers play a constantly increasing role in modern medicine. The Framingham Heart Study identified a whole set of traditional markers that are commonly accepted as predictors of cardiovascular disease. Among them we can distinguish age, gender, cigarette smoking, high blood pressure, elevated cholesterol levels, diabetes mellitus, obesity and familial history of coronary heart disease. However, there was no observable risk factor in 10-15% of patients who developed CVDs [288, 289]. On the contrary, some people with traditional risk factors do not develop CVDs. Moreover, classical risk factors are not optimal in reference to the efficacy assessment of new cardiovascular

drugs. In order to demonstrate the substantial benefit of a new therapeutic drug, clinical trials including large cohorts followed for many years have to be conducted. The lack of good biomarkers limits our ability to exclude potential therapeutics that do not meet the expected outcome at early stages of drug development.

**Table 5-1. Genes prone to signal integration are associated with CVDs.**

Genomatix software were used to assign 30 synergistically amplified genes listed in table 3-2 to the MeSH terms associated with diseases. 20 most relevant terms related to CVDs is presented.

MeSH-Term	P-value	List of observed genes
<b>Systemic Inflammatory Response Syndrome</b>	1,06E-12	Tnfaip2, Gja4, Neurl3, Gbp5, Tnfrsf11a, Rsad2, Mx1, Ccl5, Nos2, Cxcl10, Irf8, Cd40, Ccl12, Has1, Egr2, Ccl8, Cxcl9, Cd74, Ubd, Atf3
<b>Hypertension, Pulmonary</b>	1,48E-09	Tnfaip2, Gja4, Tnfrsf11a, Mx1, Ccl5, Nos2, Cxcl10, Irf8, Cd40, Has1, Cxcl9, Serpina3g, Atf3
<b>Inflammation</b>	6,31E-09	Tnfaip2, Gja4, Neurl3, Ccl12, Gbp5, Tnfrsf11a, Rsad2, Mx1, Ccl5, Nos2, Cxcl10, Irf8, Cd40, Ccl12, Has1, Egr2, Ccl8, Cxcl9, Serpina3g, Cd74, Ubd, Atf3, Ifi205
<b>Atherosclerosis</b>	4,24E-08	Tnfaip2, Gja4, Tnfrsf11a, Mx1, Ccl5, Nos2, Cxcl10, Irf8, Cd40, Has1, Egr2, Ccl8, Cxcl9, Cd74, Atf3
<b>Fibrosis</b>	1,92E-07	Tnfaip2, Gja4, Tnfrsf11a, Mx1, Ccl5, Nos2, Cxcl10, Cd40, Ccl12, Has1, Egr2, Ccl8, Cxcl9, Cd74, Atf3
<b>Autoimmune Diseases</b>	3,01E-07	Tnfaip2, Gja4, Ccl12, Tnfrsf11a, Rsad2, Mx1, Ccl5, Nos2, Cxcl10, Irf8, Cd40, Ccl12, Has1, Egr2, Ccl8, Cxcl9, Serpina3g, Cd74, Ubd, Atf3, Ifi205, Upp1
<b>Arteriosclerosis</b>	1,01E-06	Tnfaip2, Gja4, Tnfrsf11a, Mx1, Ccl5, Nos2, Cxcl10, Irf8, Cd40, Has1, Egr2, Ccl8, Cxcl9, Cd74, Atf3, Upp1
<b>Aortic Arch Syndromes</b>	1,84E-06	Tnfaip2, Tnfrsf11a, Ccl5, Nos2, Cd40
<b>Carotid Artery Diseases</b>	2,12E-06	Tnfaip2, Gja4, Tnfrsf11a, Rsad2, Ccl5, Nos2, Cxcl10, Cd40, Ccl8, Cxcl9, Cd74
<b>Arterial Occlusive Diseases</b>	2,37E-06	Tnfaip2, Gja4, Tnfrsf11a, Mx1, Ccl5, Nos2, Cxcl10, Irf8, Cd40, Has1, Egr2, Ccl8, Cxcl9, Cd74, Atf3, Upp1
<b>Myocardial Infarction</b>	5,82E-06	Tnfaip2, Gja4, Tnfrsf11a, Ccl5, Nos2, Cxcl10, Cd40, Has1, Egr2, Ccl8, Cxcl9, Cd74, Atf3
<b>Myocarditis</b>	8,65E-06	Tnfaip2, Tnfrsf11a, Ccl5, Nos2, Cxcl10, Cd40, Ccl12, Cxcl9
<b>Coronary Disease</b>	2,32E-05	Tnfaip2, Gja4, Tnfrsf11a, Ccl5, Nos2, Cxcl10, Irf8, Cd40, Has1, Ccl8, Cxcl9, Cd74
<b>Aortic Aneurysm, Abdominal</b>	2,63E-05	Tnfaip2, Mx1, Ccl5, Nos2, Cxcl10, Cd40, Cxcl9
<b>Carotid Stenosis</b>	2,63E-05	Tnfaip2, Gja4, Tnfrsf11a, Ccl5, Nos2, Cd40, Cd74
<b>Myocardial Ischemia</b>	2,77E-05	Tnfaip2, Gja4, Tnfrsf11a, Ccl5, Nos2, Cxcl10, Irf8, Cd40, Has1, Egr2, Ccl8, Cxcl9, Cd74, Ubd, Atf3
<b>Hypertension, Portal</b>	2,84E-05	Tnfaip2, Gja4, Ccl5, Nos2, Cxcl10, Cxcl9
<b>Aneurysm, Ruptured</b>	3,56E-05	Tnfaip2, Ccl5, Nos2, Cxcl10, Cxcl9
<b>Acute Coronary Syndrome</b>	5,24E-05	Tnfrsf11a, Ccl5, Nos2, Cxcl10, Cd40, Cxcl9
<b>Hyperlipidemias</b>	6,06E-05	Tnfaip2, Tnfrsf11a, Ccl5, Nos2, Cxcl10, Irf8, Cd40, Has1, Ccl8, Atf3

As a consequence, there has been a dramatic increase in R&D costs and in the pharmaceutical industry's shift of resources towards other research areas [290]. Thus, identifying new markers of clinical endpoints in CVDs is crucial for public health. Such new, validated biomarkers will help detect and monitor progression of the disease. Additionally, new

biomarkers will aid in quick identification of potential targets that are toxic or did not provide better clinical efficacy.

Recent results obtained in our laboratory as well as data presented in this thesis suggest that sets of synergistically amplified STAT1-dependent genes can be considered as novel diagnostic markers of CVDs. Indeed, by using the Medical Subject Headings Database (MeSH) [291] we could observe a significant association between 30 investigated genes (Table 3-2) and cardiovascular diseases (Table 5-1). The results of the screening confirmed statistically significant enrichment of MeSH terms related to CVDs and pointed to the role of amplified genes in CVDs. Furthermore, we used two microarray datasets obtained from human coronary plaques and human carotid plaques (acc. no. GSE40231 and GSE21545, respectively [292, 293]) and compared them with the 30 IFN $\gamma$ - and LPS-amplified STAT1-target genes. Our data mining of the microarray studies obtained from human specimens identified 12 out of 30 synergistically amplified genes to be expressed in carotid plaques and 6 out of 30 in coronary plaques (Table 5-2).

**Table 5-2. Expression of synergistically amplified genes from Table 3-2 in human atherosclerotic vessels [198]**

<b>Gene Name</b>	<b>Human carotid plaques</b>	<b>Human coronary plaques</b>
<b>CD74</b>	+	
<b>CCL5</b>	+	+
<b>CXCL10</b>	+	+
<b>GBP5</b>	+	
<b>IRF8</b>	+	
<b>CCL8</b>	+	
<b>CXCL9</b>	+	+
<b>CCRL2</b>	+	
<b>UBD</b>	+	
<b>SECTM1</b>	+	
<b>IFI16</b>	+	
<b>UPP1</b>	+	+
<b>ATF3</b>		+
<b>FAM26F</b>		+

Additional studies from our group predicted potential STAT1 but also STAT1-NFκB and STAT1-IRF modules in many of the genes expressed in these types of plaques [198]. These genes were involved in processes that are crucial for formation of the plaque, such as cell adhesion, migration, matrix remodeling and calcification. Importantly, many of the potentially STAT1-dependent proteins are either membrane-bound or secreted, and as such can be detected in the serum [198]. Therefore, it is tempting to suggest a selection of these genes as markers of the onset of atherosclerosis. Studies with the multi-marker approach using the above identified STAT1-dependent genes may reveal a substantial clinical benefit. Although further research is needed to confirm our hypothesis, data provided by other groups seem to support it; for example, Harder et al. showed that in addition to traditional risk factors, 13 inflammatory markers (including STAT1-dependent ones such as CXCL10, CCL2, CCL5) significantly improved the prediction of coronary events and type 2 diabetes [294]. Kharti et al. analyzed microarray studies from 236 graft biopsy samples from four different organs and identified 11 genes (e.g. *CXCL10*, *CXCL9*,) overexpressed in acute rejection [295]. More importantly, they found that STAT1 and NFκB are central regulators of 10 identified genes and that their expression correlates with the degree of organ damage. Then they confirmed that STAT1- and NFκB-dependent genes are expressed in the animal model of the heart transplant and showed that treatment with atorvastatin reduces expression of these genes and as such is beneficial for allograft survival [295]. It is tempting to suggest a similar approach in studying the role of STAT1, IRFs and genes regulated by their activity in different models of CVDs.

## Conclusions

Taken together, our results provide further evidence for the crosstalk between IFN $\gamma$  and TLR4 in non-immune cells and indicate the central role of STAT1, activated IRF1 and IRF8 in the mechanism underlying expression of proinflammatory mediators. A genome-wide analysis in VSMCs identified a whole set of STAT1-dependent genes that were synergistically affected by interactions between IFN $\gamma$  and TLR4. Among the highly amplified genes we could predominantly distinguish chemokines and adhesion molecules. Functional assays together with immunohistochemical stainings of phosphorylated STAT1 and STAT1-dependent genes confirmed the importance of STAT1 in the regulation of vascular inflammation. Moreover, analysis of STAT1 function in Ang II-induced hypertensive end organ damage further supported its role in the regulation of inflammation and vessel function. These data provide new insight into understanding the role of STAT1-driven inflammatory processes which, in turn, play a crucial role in the pathophysiology of CVDs.

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## Important Abbreviations

Ang II	-angiotensin II
BP	- blood pressure
CVDs	- cardiovascular diseases
DAMPs	- damage-associated molecular patterns
DCs	- dendritic cells
ECs	- endothelial cells
GAS	- interferon-gamma-activated sequence
HMECs	- human microvascular endothelial cells
HSPs	- heat shock proteins
IFN	- interferon
IL	- interleukin (eg. IL-6)
ISRE	- interferon stimulated response element
LPS	- lipopolysaccharide
IRFs	- Interferon regulatory factors
JAK1	- janus kinase 1
JAK2	- janus kinase 2
LDL	- low-density lipoprotein
NFκB	- nuclear factor kappa B
NGAL	- neutrophil gelatinase-associated lipocalin
oxLDL	- oxidized low-density lipoprotein
PAMPs	- pathogen-associated molecular patterns
PRRs	- pathogen recognition receptors
ROS	- reactive oxygen species
SHR	- spontaneously hypertensive rates
SMCs	- smooth muscle cells
STAT1	- signal transducer and activator of transcription 1
TLRs	- toll-like receptors (eg. TLR4)
TNFα	- tumor necrosis factor alpha
TRIF	- TIR-domain-containing adapter-inducing interferon-β
VSMCs	- vascular smooth muscle cells
WT	- wild type

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## Streszczenie w języku polskim

Najnowsze badania z zakresu etiologii chorób układu krążenia wskazują na negatywną rolę układu immunologicznego. W specyficznych warunkach środowiskowych ściśle powiązanych ze stylem życia, układ immunologiczny może przyczyniać się do rozwoju takich chorób jak miażdżycza czy nadciśnienie tętnicze. Wraz z aktywacją systemu odporności dochodzi do uszkodzenia śródbłonna, co z kolei skutkuje osłabieniem zdolności regulowania wazodylatacji. Dysfunkcja komórek budujących naczynia krwionośne związana jest również z ekspresją substancji prozapalnych (m.in. chemokin, cytokin i cząsteczek adhezyjnych), które umożliwiają migrację oraz adhezję komórek układu immunologicznego do ściany naczynia.

Istotną rolę w regulacji procesów zapalnych odgrywa interferon gamma (IFN $\gamma$ ) oraz czynniki wpływające na receptor Toll-podobny 4 (TLR4). IFN $\gamma$  stymuluje szlak sygnałowy JAK/STAT poprzez aktywację czynnika transkrypcyjnego STAT1 oraz czynników transkrypcyjnych regulowanych interferonem (IRF). Doświadczenia przeprowadzone na komórkach układu immunologicznego wykazały, że zarówno szlak przekazywania sygnału JAK/STAT, jak i TLR4 współdziałają ze sobą za pośrednictwem białek STAT1 oraz białek z rodziny IRF. Eksperymenty przedstawione w tej pracy miały na celu zweryfikowanie funkcji czynnika transkrypcyjnego STAT1 oraz IRF w integracji szlaków sygnalizacyjnych IFN $\gamma$  i receptora TLR4 w aktywacji procesów zapalnych związanych z chorobami układu krążenia.

Nasza hipoteza zakładała, że w komórkach nienależących do układu immunologicznego, takich jak komórki śródbłonna oraz mięśni gładkich, integracja szlaków sygnalizacyjnych JAK/STAT oraz TLR4 za pośrednictwem STAT1 oraz IRF prowadzi do synergistycznego wzrostu ekspresji białek zaangażowanych w proces zapalny. W pierwszym rozdziale podsumowano aktualną wiedzę na temat szlaków sygnalizacyjnych JAK/STAT, TLR4 oraz czynników transkrypcyjnych STAT1 oraz IRF ze szczególnym uwzględnieniem ich roli w chorobach układu krążenia. Rozdział drugi zawiera serię eksperymentów na komórkach śródbłonna, mięśni gładkich oraz komórek budujących kanaliki nerkowe, które udowadniają istnienie integracji szlaków sygnalizacyjnych JAK/STAT oraz TLR4 za pośrednictwem STAT1 oraz IRF. Eksperymenty omówione w trzecim rozdziale nie tylko identyfikują grupy genów podatnych na integrację wyżej wymienionych szlaków sygnalizacyjnych, ale również dostarczają dowodów na jej funkcjonalne znaczenie w patogenezie chorób układu krążenia. Ponadto rozdział ten opisuje nowo zidentyfikowany

czynnik transkrypcyjny IRF8 oraz wskazuje jego potencjalną rolę. W kolejnej części weryfikowana jest funkcja STAT1 w modelu nadciśnienia indukowanego angiotensyną II. Poprzez analizę eksperymentów wykonanych na zwierzętach pozbawionych genu STAT1 wykazano istotną funkcję białka STAT1 w mechanizmie ekspresji genów związanych z procesem zapalnym oraz białek uczestniczących w indukcji stresu oksydacyjnego. W ostatnim rozdziale podsumowane zostały wyniki doświadczeń oraz przeanalizowane wady i zalety potencjalnych możliwości zastosowania związków wpływających na aktywność STAT1 oraz potencjalne zastosowanie w diagnostyce chorób układu krążenia.

Badania przeprowadzone w tej pracy poszerzyły wiedzę z zakresu etiologii chorób układu krążenia takich jak miażdżyca i nadciśnienie tętnicze. Wykonane eksperymenty potwierdziły istnienie mechanizmu kooperacji pomiędzy szlakiem JAK/STAT i TLR4 w komórkach nieimmunologicznych, wskazując jednocześnie na kluczową rolę białek STAT1 oraz IRF

## Supplement

### List of publications

STAT1-mediated signal integration between IFN $\gamma$  and LPS leads to increased EC and SMC activation and monocyte adhesion - Sikorski K., Chmielewski S., Przybyl L., Heemann U., Wesoly J., Baumann M., Bluysen H.A.R – American Journal of Physiology: Cell Physiology, vol. 300, pp. C1337-1344, 2011 - **enclosed in supplement**

Immuno-suppression With 4SC-101, a Novel Inhibitor of Dihydroorotate Dehydrogenase, in a Rat Model of Renal Transplantation - Rusai, K., Schmaderer C., Baumann M., Chmielewski S., Prókai A., Kis E. et al. - Transplantation 93(11): 1101-1107, 2012

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## STAT1-mediated signal integration between IFN $\gamma$ and LPS leads to increased EC and SMC activation and monocyte adhesion

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**Sikorski K, Chmielewski S, Przybyl L, Heemann U, Wesoly J, Baumann M, Bluysen HA.** STAT1-mediated signal integration between IFN $\gamma$  and LPS leads to increased EC and SMC activation and monocyte adhesion. *Am J Physiol Cell Physiol* 300: C1337–C1344, 2011. First published February 23, 2011; doi:10.1152/ajpcell.00276.2010.—Inflammation plays an important role in host defenses against infectious agents and injury, but it also contributes to the pathophysiology of atherosclerosis. Signal transducer and activator of transcription 1 (STAT1) has been identified as a point of convergence for the cross talk between the pro-inflammatory cytokine interferon  $\gamma$  (IFN $\gamma$ ) and the Toll-like receptor-4 (TLR4) ligand LPS in immune cells. However, there is no information available on the role of STAT1 in TLR4-mediated progression of atherosclerosis and on potential synergism between lipopolysaccharides (LPS) and IFN $\gamma$  signaling in cells from the vasculature. Cultured human microvascular endothelial cells (HMECs) exposed to LPS activated STAT1 in a delayed manner that was inhibited by cycloheximide treatment. Pretreatment of HMECs as well as primary vascular smooth muscle cells (VSMCs) with IFN $\gamma$  followed by LPS resulted in a significant increase in STAT1 phosphorylation compared with both factors alone. Increased STAT1 protein levels, strictly mediated by IFN $\gamma$ , correlated with the augmented STAT1 phosphorylation that was absent in TLR4<sup>-/-</sup> cells. As assessed by PCR, Western analysis, and ELISA, this coincided with increased expression of the chemokine interferon gamma-induced protein 10 kDa (IP-10) and the adhesion molecule ICAM-1 in a TLR4-dependent manner. The STAT1-inhibitor fludarabine markedly reduced these effects as well as IFN $\gamma$  and LPS-dependent adhesion of U937 cells to endothelial cells, emphasizing the potential importance of STAT1 in the integration of both signals. With the established roles of IFN $\gamma$  and TLRs in atherosclerotic pathology, the STAT1-dependent signal integration between IFN $\gamma$  and TLR in ECs and VSMCs in response to exogenous and endogenous atherogenic ligands could result in amplification of pro-inflammatory responses in the damaged vessel and be a novel mechanism involved in the initiation and progression of atherosclerosis.

interferon- $\gamma$ ; lipopolysaccharide; signal transducer and activator of transcription 1; inflammation; atherosclerosis

INFLAMMATION participates importantly in host defenses against infectious agents and injury, but it also contributes to the pathophysiology of atherosclerosis. Endothelial cells (ECs) play a crucial role in maintaining normal vessel wall function by their ability to inhibit thrombus formation, leukocyte adhesion, vascular smooth muscle cell (VSMC) proliferation, and by regulation of vessel tone. Pro-inflammatory stimuli trigger the endothelium to produce cell surface adhesion molecules,

chemokines, and inflammatory cytokines, characteristic parameters of endothelial dysfunction (12). Subsequent recruitment of blood leukocytes to the injured vascular endothelium and VSMC proliferation characterizes the initiation and progression of atherosclerosis (28).

The pro-inflammatory cytokine interferon (IFN) $\gamma$ , derived from T cells and natural killer cells (NK cells), is vital for both innate and adaptive immunity by activating macrophages, NK cells, B cells, ECs, and VSMCs. Evidence that IFN $\gamma$  is necessary and sufficient to cause vascular remodeling is supported by mouse models of atheroma, as the serological neutralization or genetic absence of IFN $\gamma$  markedly reduces the extent of atherosclerosis (18). In addition, IFN $\gamma$  induces chemokine production, adhesion, apoptosis, and matrix deposition (30). The signal transduction pathway initiated by binding of IFN $\gamma$  to its receptor leads to rapid intracellular phosphorylation and activation of signal transducer and activator of transcription (STAT)1 (35). As such, IFN $\gamma$  is considered to participate in promoting atherogenic responses through STAT1-mediated “damaging” signals, which play a major role in driving the immune and pro-inflammatory responses leading to EC dysfunction, VSMC proliferation, and vascular damage (32).

More recent studies in macrophages and dendritic cells have also revealed that STAT1 is involved in the signaling events mediated by Toll-like receptor 4 (TLR4), which is an innate immune pattern recognition receptor that is activated by lipopolysaccharides (LPS) (1). TLR4 is expressed on a variety of cells, including ECs and VSMCs, and thus initiates and sustains the inflammatory response in atherosclerosis (2). TLR4 signaling leads to the induction of various target genes that include those encoding type I interferons (IFNs), pro-inflammatory cytokines, chemokines, and cell surface molecules. Some of these genes are regulated in a delayed manner, which is mediated by the induction and subsequent autocrine activities of type I IFN in a STAT1-dependent manner (14).

Consequently, STAT1 has been identified as a point of convergence for the cross talk between IFN $\gamma$  and LPS in immune cells (25), which is considered relevant for the efficient induction of inflammatory mediators to eliminate pathogens. However, there is no information available on the role of STAT1 in TLR4-mediated progression of atherosclerosis and on potential synergism between LPS- and IFN $\gamma$ -signaling in cells from the vasculature.

We provide evidence that in ECs and VSMCs in vitro STAT1-dependent functions of IFN $\gamma$  supply a platform for increased LPS signaling, resulting in augmented STAT1 phosphorylation and expression of intercellular adhesion molecule (ICAM)-1 and the chemokine interferon gamma-induced protein 10 kDa (IP-10). Fludarabine, a purine analog with immu-

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nosuppressive activity through the specific inhibition of STAT1 signaling (9, 32), markedly reduced these effects as well as IFN $\gamma$  and LPS-dependent adhesion of monocytes to ECs.

#### MATERIALS AND METHODS

**Reagents.** Recombinant IFN $\gamma$  was purchased from Millipore, and LPS (*Escherichia coli* 0111:B4) was from Sigma. Cycloheximide was from Bioshop (Canada). Fludarabine was kindly provided by Dept. of Hematology, Medical University, Poznan, Poland.

Rabbit polyclonal antibodies against p-Tyr (701) STAT1, STAT1, interferon regulatory factor 1 (IRF1), IP-10, and ICAM-1 and mouse antibody against GAPDH and horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology.  $\alpha$ -Tubulin antibody was purchased from Sigma.

**Cell culture.** Human microvascular endothelial cells (HMEC) were generously provided by the Center for Disease Control and Prevention (Atlanta, GA) and cultured as described (3) in MCDB-131 (IITD PAN, Wroclaw, Poland) medium containing 10% fetal calf serum (FCS) (Invitrogen), 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, 0.01  $\mu$ g/ml EGF, 0.05  $\mu$ M hydrocortisone, and 2 mM L-glutamine; MCDB-10%. Human leukemia U937 suspension cells were cultured in DMEM (IITD PAN) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and 10% FBS. Mouse aortic VSMC were cultured in DMEM (Biochrom) medium containing 10% FBS (Sigma-Aldrich, Munich, Germany) and 2 mM L-glutamine (Biochrom) supplemented with 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin. VSMC were isolated from C57BL/6 or TLR4<sup>-/-</sup> mice using the protocol according to Kobayashi et al. (16). Homogeneity of the culture was assessed by the expression of  $\alpha$ -smooth muscle actin (5'-CAACTGGTATTGTGCTGGACT-3'; 5'-GAAAGATGGCTGGAAGAGAGT-3'), calponin (5'-ACGGCTTGCTGCTGAAGTA-3'; 5'-AAGATGTCTGGTGGGTTTAC-3'), and smoothelin (5'-AGAACTGGCTACACTCTCAAC-3'; 5'-GGGTCCAATGTGTGTGCTG-3').

In general, HMEC or VSMC treatment with 10 ng/ml of IFN $\gamma$  and/or 1  $\mu$ g/ml of LPS, as well as 75  $\mu$ g/ml of cycloheximide and 100  $\mu$ M fludarabine, was performed in serum-starved medium (containing 2% serum) after starvation of at least 16 h before an experiment.

From wild-type (WT) and TLR4<sup>-/-</sup> VSMCs ( $n = 4$ ), RNA was isolated using RNeasy Mini Kit (QIAGEN, Hilden, Germany) and transcribed into cDNA with iScript cDNA synthesis kit (Bio-Rad) following the manufacturer's protocols. From HMECs total RNA was isolated using GeneMATRIX Universal RNA Purification Kit (Eurx, Gdansk, Poland). For RT 500 ng of total RNA was used and transcribed using RevertAid First Strand cDNA synthesis kit (Fermentas) and random hexamer primers.

**Western blot analysis.** HMECs were lysed using RIPA buffer (50 mM Tris-HCl, pH = 8.0, 150 mM NaCl, 1% Nonidet-40, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitor cocktail), VSMCs using Lysis-M Reagent (Roche) containing protease and phosphatase inhibitors (Roche), and stored at -80°C. Twenty micrograms of protein were boiled with sample buffer (except for ICAM-1; samples were run under nonreducing conditions) and loaded on a 10% acrylamide gel, electrophoresed, and transferred onto PVDF membranes (generally, 0.45  $\mu$ m pore size was used, except for IP-10 0.2  $\mu$ m) using a semidry transfer system (Hoefer). Membranes were blocked in 5% nonfat dry milk in TBS-Tween (TBS-T) and incubated with primary antibodies (1:200 pSTAT1, 1:500 STAT1, 1:200 IRF1, 1:100 IP-10, 1:200 ICAM-1, 1:2,000 GAPDH, 1:200  $\alpha$ -tubulin). After being washed three times in TBS-T, the membrane was incubated with goat anti-mouse or goat anti-rabbit HRP-conjugated (1:20,000) antibody and then washed three times in TBS. Bound proteins were detected with Immobilon Western chemiluminescent substrate (Millipore). Membranes were stripped with buffer containing 25 mM glycine and 1% SDS, pH 2.0, and then reprobed. The intensity of each

band was quantified using Photoshop and normalized to the level of GAPDH protein. Results are expressed as means (P-STAT1/STAT1, P-STAT1/GAPDH, STAT1/GAPDH, IP-10/GAPDH, or ICAM1/GAPDH)  $\pm$  SE of at least three independent experiments.

**ELISA.** Murine IP-10 ELISA development kit was obtained from Peprotech (Hamburg, Germany) and was used according to the manufacturer's instruction.

**Gene expression analyses by qPCR and PCR.** QPCR on VSMCs was performed using a MyiQ Real Time PCR detection system provided by Bio-Rad. Data were normalized to the level of GAPDH expression and were evaluated in Bio-Rad iQ5 software. The primers used were the following: GAPDH, 5'-TCGGTGTGAACGGATTGGC-3' and 5'-TGATGTTAGTGGGCTCTCGC-3'; IP-10, 5'-TCATCCCTGCGAGCCTATCC-3' and 5'-GGAGCCCTTTAGACCTTTT-3'.

For qPCR experiments conducted on HMECs (see Fig. 2B), 7900HT real-time PCR system (Applied Biosystems) and Maxima SYBR Green/ROX qPCR MasterMix (Fermentas) were used. The amount of target gene in each subject was normalized to endogenous control ACT- $\beta$  ( $\Delta$ CT). Gene expression is expressed as means  $\pm$  SE of three independent experiments. Data were transformed as described (36). The primers used were the following: ACT- $\beta$ , 5'-ACAGAGCCTCGCCTTTCGCGAT-3' and 5'-ATCATCCATGGTGAGCTGGCGG-3'; IP-10, 5'-GCAGAGGAACCTCCAGTCTCAGCA-3' and 5'-AGAGAGAGG-TACTCCTTGAATGCCAC-3'; ICAM-1, 5'-CAGCGGTGACGTGTGCAGTAA-3' and 5'-TTGGGCGCCGGAAGCTGTA-3'.

Regular (semiquantitative) PCR (see Figs. 1C and 3D) was conducted using Allegro Taq (Novazym, Poznan, Poland). Primers used were as follows: 18S, 5'-AGTTGGTGGAGCGATTTGTC-3' and 5'-TATTGCTCAATCTCGGGTGG-3'; IP-10, 5'-GACTCTAAGTGGCATTCAAG-3' and 5'-TCAGACATCTCTTCTCACC-3'; ICAM-1, 5'-TGACCATCTACAGCTTTCCG-3' and 5'-TCTTGTGTATAAGCTGCCG-3'.

**Adhesion assay.** HMEC cells were seeded onto six-well plates and grown to 80–90% confluence. The cells were starved for at least 16 h. Cells were treated with IFN $\gamma$  and LPS as described above. The cells were left on the plates for 12 h after treatment. U937 cells were labeled with MTT according to Miki et al. (20), albeit the cells were incubated with MTT for 1.5 h. After labeling was completed,  $1 \times 10^6$  cells were resuspended in 2 ml of fresh medium and added to each well of treated HMECs, from which the medium was removed. After 45 min of incubation at 37°C, cells were washed with PBS and 1 ml of DMSO was added and incubated for another 20 min. Next, the absorbance was measured at 540 nm using Lambda EZ150 (Perkin Elmer) spectrophotometer. A calibration curve of various amounts of labeled U937 cells (5, 15, 45, 150, 500  $\times 10^3$ ) was used to calculate the number of adhered cells. Data are expressed as a percentage of basal adhesion of U937 cells to untreated HMECs.

**Data analysis.** Results are expressed as means  $\pm$  SE. Data were compared by a one-way ANOVA and the Tukey HSD post hoc test, when appropriate. All statistical tests were performed with Statistica software (Statsoft);  $P < 0.05$  was considered significant.

#### RESULTS

**LPS-induced activation of STAT1 and target gene expression in endothelial cells.** Treatment of HMECs with LPS resulted in STAT1 phosphorylation in a time-dependent fashion (Fig. 1A) and dose-dependent fashion (not shown). STAT1 phosphorylation was only detectable after 4 h of exposure to 1  $\mu$ g/ml LPS (not using 0.1 or 0.3  $\mu$ g/ml; not shown), decreased after 8 h, and was hardly detectable at 24 h. LPS did not affect total amounts of STAT1 protein at these time points. In contrast, IFN $\gamma$ -induced STAT1 phosphorylation in HMECs occurs rapidly (within 30 min) and results in a time-dependent increase of STAT1 protein (see Ref. 3 and data not shown).

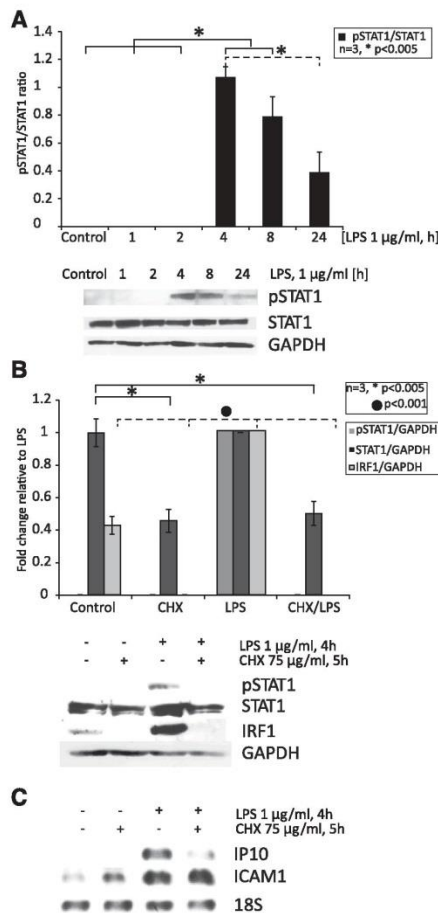


Fig. 1. Lipopolysaccharide (LPS)-induced signal transducer and activator of transcription 1 (STAT1) signaling in endothelial cells (ECs). Human microvascular endothelial cells (HMECs) were treated with 1  $\mu$ g/ml of LPS (A) for different time points or pretreated with 75  $\mu$ g/ml of cycloheximide (CHX) for 1 h and then treated with LPS for 4 h (B and C). A and B: protein extracts were analyzed by Western blot analysis for tyrosine phosphorylated STAT1, STAT1, and GAPDH. C: RNA was isolated and subjected to RT-PCR for interferon gamma-induced protein 10 kDa (IP-10) and ICAM-1. For all experiments  $n = 3$ , graphs represent mean intensity of Western blot bands  $\pm$  SE. Data were tested for significance by one-way ANOVA, followed by post-hoc Tukey HSD test. See text for more details.

Next, we treated HMECs with LPS for 4 h in the absence or presence of cycloheximide (pretreated for 1 h). Figure 1B shows that LPS-induced STAT1 phosphorylation (lane 3) as well as expression of the STAT1-target IRF1 is blocked in the presence of cycloheximide (lane 4), which correlates with a protein synthesis-dependent mechanism. Indeed, LPS-dependent IFN $\beta$  expression preceded STAT1 phosphorylation (data

not shown). STAT1 protein expression, however, did not change under these conditions (lanes 3 and 4).

Following expression of the known IFN $\gamma$ :STAT1-target genes, IP-10 and ICAM-1 (3, 23), under these conditions (Fig. 1C) unraveled a protein synthesis-dependent effect for IP-10 and a potential protein synthesis-independent effect for ICAM-1 (lanes 3 and 4).

IFN $\gamma$  sensitizes ECs for LPS-induced STAT1-phosphorylation and target gene expression. Next, we studied the possibility of STAT1-dependent cross talk in ECs between LPS and IFN $\gamma$ . Based on the IFN $\gamma$  and LPS time-course experiments (see above), we decided to use the IFN $\gamma$  for 8 h (10 ng/ml) and LPS for 4 h (1  $\mu$ g/ml) to study the effect of IFN $\gamma$  pretreatment on LPS signaling. Indeed, we observed that pretreatment of HMECs with IFN $\gamma$  for 4 h followed by LPS for another 4 h (see Fig. 2A) resulted in a significant increase in STAT1 phosphorylation compared with both factors alone (compare lane 4 with 2 and 3). Increased STAT1 protein levels, strictly dependent on IFN $\gamma$  (Fig. 2A; lanes 4, 2, 3), could provide a possible explanation for the augmented STAT1 phosphorylation under conditions when both IFN $\gamma$  and LPS are present.

Similarly, IP-10 and ICAM-1 were identified as IFN $\gamma$ - as well as LPS-inducible genes, which coincided with STAT1 phosphorylation. Moreover, increased mRNA (Fig. 2B) and protein (Fig. 2C) levels of both IP-10 and ICAM-1 could be detected in HMECs pretreated with IFN $\gamma$  for 4 h followed by LPS for another 4 h, compared with both factors alone (compare lane 4 with 2 and 3).

In ECs STAT1-mediated cross talk between IFN $\gamma$  and LPS is protein-synthesis dependent. As can be depicted from Fig. 3A, the increase in STAT1 phosphorylation observed in HMECs treated with IFN $\gamma$  for 4 h followed by LPS for 4 h (lanes 1–4) is blocked in the presence of cycloheximide (lanes 5–8), which correlates with a protein synthesis-dependent mechanism. More important, cycloheximide also inhibited IFN $\gamma$ -mediated increase in STAT1 protein levels (Fig. 3A; lanes 1–4, 5–8), providing additional evidence that augmented STAT1 phosphorylation under conditions when both IFN $\gamma$  and LPS are present is dependent on STAT1 protein synthesis.

Fludarabine attenuates STAT1-dependent cross talk between IFN $\gamma$  and LPS and expression of IP-10 and ICAM-1. To obtain further evidence for a direct role of STAT1 in cross talk between IFN $\gamma$  and LPS, we first treated HMECs with IFN $\gamma$ , LPS, or both in the absence or presence of the STAT1-inhibitor fludarabine. As such, fludarabine was able to partially prevent the augmented effect of IFN $\gamma$  on LPS-induced STAT1 phosphorylation and STAT1 expression but almost completely abolished IRF1 expression (Fig. 3B; lanes 1–4, 5–8).

Fludarabine also affected gene expression of IP-10 and ICAM-1 in HMECs treated with IFN $\gamma$ , LPS, or both, although in different ways (Fig. 3C). The increase in IP-10 gene expression (Fig. 3C) expression observed in HMECs treated with IFN $\gamma$  followed by LPS (lanes 1–4) was almost completely absent in the presence of fludarabine (lanes 5–8), which coincided with IP-10 protein levels (Fig. 3D) and correlates with a predominant STAT1-dependent mechanism. On the other hand, the increased gene expression of ICAM-1 (Fig. 3C) was affected by fludarabine to a lesser extent, implicating only partial involvement of STAT1. Although ICAM-1 protein levels were rather low under these conditions, a comparable expression pattern could be detected (Fig. 3D).



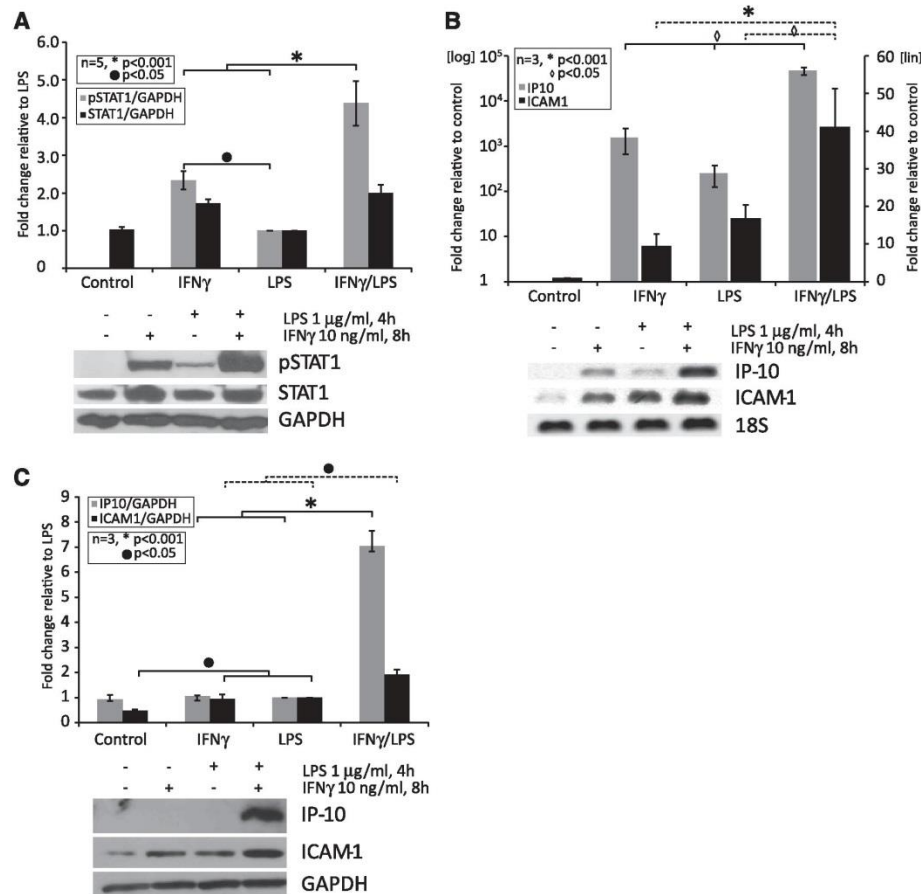


Fig. 2. Effects of cross talk between interferon  $\gamma$  (IFN $\gamma$ ) and LPS in ECs. **A**: HMECs were treated with 10 ng/ml of IFN $\gamma$  for 8 h and/or 1  $\mu$ g/ml of LPS for 4 h. Protein extracts were analyzed by Western blot analysis for tyrosine-phosphorylated STAT1, total STAT1, and GAPDH. **B**: HMECs were treated as in **A**. RNA was isolated and subjected to qRT-PCR for IP-10 and ICAM-1. **C**: cells were treated as in **A** and **B**. Protein extracts were analyzed by Western blot analysis for IP-10 and ICAM-1 proteins. For all experiments  $n \geq 3$ , graphs represent mean intensity of Western blot bands  $\pm$  SE (**A** and **C**) or relative mean mRNA level  $\pm$  SE (**B**). Data were tested for significance by one-way ANOVA, followed by post-hoc Tukey HSD test. See text for more details.

In VSMCs STAT1-mediated cross talk between IFN $\gamma$  and LPS is TLR4 dependent. In analogy to ECs (Fig. 2A), we observed that pretreatment of VSMCs with IFN $\gamma$  for 4 h followed by LPS for another 4 h (see Fig. 4A) also resulted in a significant increase in STAT1 phosphorylation compared with both factors alone (compare lane 4 with 2 and 3). Increased STAT1 protein levels, as in EC, were strictly dependent on IFN $\gamma$  (Fig. 4A; compare lane 4 with 2 and 3). Moreover, in TLR4 $^{-/-}$  VSMCs, LPS was not able to phosphorylate STAT1 (Fig. 4A; lane 7). As a consequence STAT1 phosphorylation was not increased under conditions when both IFN $\gamma$  and LPS were present, even though STAT1 expression was still upregulated by IFN $\gamma$  (lanes 5–8).

mRNA levels of IP-10 in VSMCs followed the expression pattern of ECs, as they were upregulated by both IFN $\gamma$  and LPS and highly increased in the presence of both agents (see Fig. 4B; lanes 1–4), coinciding with phosphorylated STAT1. Moreover, this happened in a TLR4-dependent fashion (lanes 5–8). Under the same conditions, IP-10 protein secreted in the medium correlated with IP-10 mRNA levels (Fig. 4C). Together, this implies that in both ECs and SMCs STAT1 provides a platform for cross talk between IFN $\gamma$  and LPS, which results in increased expression of pro-inflammatory target genes.

Fludarabine inhibits IFN $\gamma$ - and LPS-dependent adhesion of monocytes to ECs. Furthermore, we investigated whether the cross talk between IFN $\gamma$  and LPS in ECs would lead to

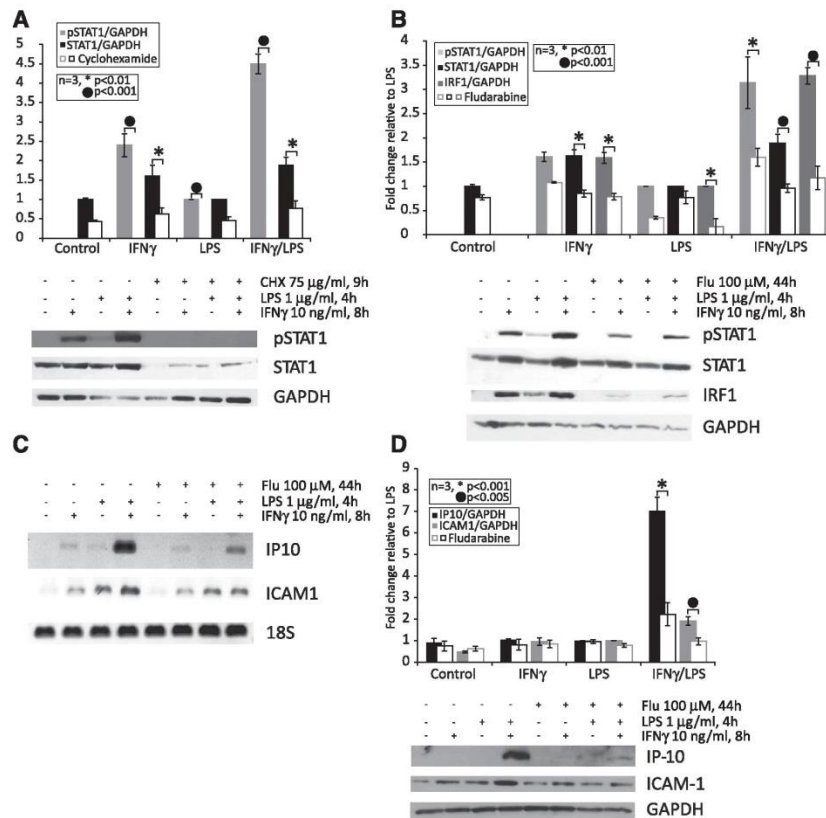


Fig. 3. Cross talk between IFN $\gamma$  and LPS in ECs is inhibited in the presence of cycloheximide and fludarabine. HMECs were treated with 10 ng/ml of IFN $\gamma$  for 8 h or 1  $\mu$ g/ml of LPS for 4 h, or both or pretreated with 75  $\mu$ g/ml of CHX for 1 h (A) or (B, C and D) pretreated with 100  $\mu$ M fludarabine for 36 h and then treated as above. Protein extracts were analyzed by Western blot analysis for tyrosine phosphorylated STAT1, total STAT1, interferon regulatory factor 1 (IRF1), and GAPDH. C: HMECs were treated as in B and RNA was subjected to RT-PCR for IP-10 and ICAM-1. D: cells were treated as in B. Protein extracts were analyzed by Western blot analysis for IP-10, ICAM-1, and GAPDH proteins. For all experiments  $n = 3$ , graphs represent mean intensity of Western blot bands  $\pm$  SE. Data were tested for significance by one-way ANOVA, followed by post hoc Tukey HSD test. See text for details.

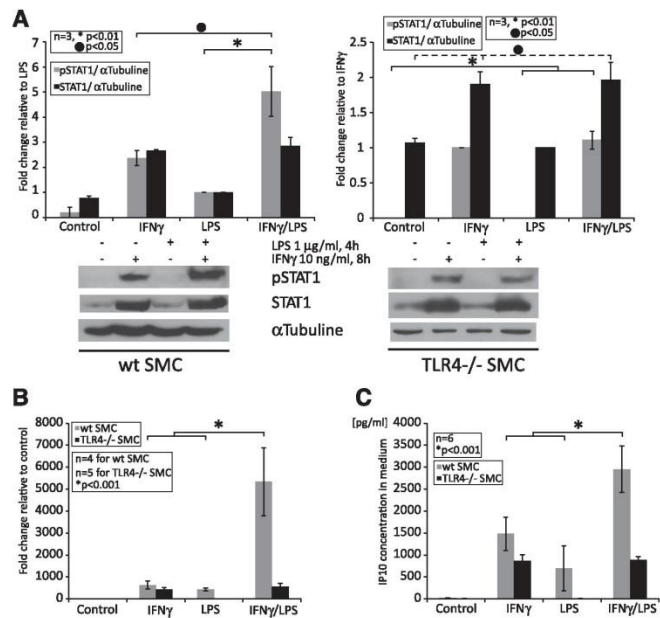
increased adhesion of monocytes to ECs. Figure 5A shows that pretreatment of HMECs with IFN $\gamma$  for 4 h followed by LPS for another 4 h resulted in a significant increase in adhesion of U937 cells compared with both factors alone. As such, IFN $\gamma$  increased adhesion by about 15% and LPS alone by 50%. But when ECs were treated with both, adhesion of monocytes increased by 120% (Fig. 5A). Interestingly, in the presence of fludarabine, adhesion of monocytes to HMECs, induced by treatment with IFN $\gamma$  followed by LPS, is decreased more than 70% (Fig. 5B). This is indicative of a STAT1-dependent mechanism.

#### DISCUSSION

IFN $\gamma$  is vital for both innate and adaptive immunity and is also expressed at high levels in atherosclerotic lesions. As

such IFN $\gamma$  plays a crucial role in the pathology of atherosclerosis through activation of STAT1. More recently, STAT1 has been identified as a point of convergence for the cross talk between IFN $\gamma$  and TLRs in immune cells (25). To date, there is limited information available on the role of STAT1 in TLR4-mediated progression of atherosclerosis and on potential synergism between LPS and IFN $\gamma$  signaling in cells from the vasculature. In a recent expression profiling study of IFN $\gamma$ -treated ECs, we identified the chronic inflammation indicators IP-10 and ICAM-1 among the highest IFN $\gamma$ -inducible genes (3), and we were interested how these genes would behave in the signal integration between IFN $\gamma$  and LPS. Our results suggest that also in ECs and VSMCs STAT1 provides a platform for cross talk between IFN $\gamma$  and LPS, which results in STAT1-dependent EC and VSMC

Fig. 4. Effects of cross talk between IFN $\gamma$  and LPS in wild-type (WT) and TLR4 $^{-/-}$  VSMCs. WT or TLR4 $^{-/-}$  VSMCs were treated with 10 ng/ml of IFN $\gamma$  for 8 h or 1  $\mu$ g/ml of LPS for 4 h, or both. A: protein extracts were analyzed by Western blot analysis for pSTAT1, total STAT1, and GAPDH. B: RNA was isolated and subjected to qRT-PCR for IP-10. C: medium was subjected to ELISA for IP-10 protein concentration. For all experiments  $n \geq 3$ , graphs represent mean intensity of Western blot bands  $\pm$  SE (A), mean mRNA level  $\pm$  SE (B), and mean concentration [pg/ml] of IP-10 in medium  $\pm$  SE. Data were tested for significance by one-way ANOVA, followed by post hoc Tukey HSD test. See text for details.



activation, increased expression of ICAM-1 and IP-10, and monocyte adhesion.

IFN $\gamma$  produced by T cells and NK cells is considered to enhance TLR signaling in DCs and macrophages for the efficient induction of inflammatory mediators to eliminate pathogens (25). IFN $\gamma$  signaling is largely mediated by the latent cytosolic factor STAT1 that is activated during IFN $\gamma$ -dependent JAK-STAT signaling (7, 35). STAT1 activity requires phosphorylation at tyrosine701 (Y701) for dimerization and induction of IFN $\gamma$ -dependent gene regulation. Typically, in macrophages LPS triggers STAT1 phosphorylation at Y701 in a delayed manner, which is mediated by the induction and subsequent autocrine activities of type I IFN (14). This coincides with our findings in ECs and VSMCs, in which LPS-induced STAT1 phosphorylation is secondary to LPS-induced IFN- $\beta$  (not shown) and abrogated in the presence of cycloheximide, which is indicative of a protein synthesis-dependent mechanism. In general it is believed that the transactivator ability of the macrophage STAT1 pool is superactivated upon stimulation with both IFN $\gamma$  and LPS, relative to either agonist alone (25). Likewise, pretreatment of ECs as well as VSMCs with IFN $\gamma$  followed by LPS resulted in a significant increase in STAT1 phosphorylation compared with both factors alone. This was dependent on protein synthesis and did not occur in TLR4 $^{-/-}$  VSMCs. Moreover, increased production of STAT1 protein in these cells, strictly dependent on IFN $\gamma$ , provides a potential mechanism resulting in augmented STAT1 phosphorylation under conditions when both IFN $\gamma$  and LPS are present. IP-10 (also denoted as CXCL10) is a monocyte and T-lymphocyte chemokine that is constitutively expressed at low levels in thymic, splenic, and lymphnode stroma; however,

expression can be highly induced by interferons in leukocytes, such as monocytes and macrophages, as well as in nonleukocytes, including ECs and VSMCs (10, 11). IP-10 was also shown to potentiate leukocyte adhesion to endothelium (29) and to be a potent mitogenic and chemotactic factor for VSMCs (34). A growing body of evidence suggests that IP-10 may play a role in chronic inflammatory diseases, including coronary artery disease and related manifestations of atherosclerosis (13, 19).

ICAM-1 is an immunoglobulin-like cell adhesion molecule expressed by several cell types including leukocytes and ECs and VSMCs (17). ICAM-1 is induced in response to inflammatory cytokines and enables EC monocyte as well as EC lymphocyte interactions. The expression of ICAM-1 has also been detected on VSMCs, indicating ability to interact with leukocytes by this mechanism (6). As such, ICAM-1 is critical for the transmigration of leukocytes out of blood vessels and into tissues. More important, ICAM-1 is present in atherosclerotic lesions and is involved in their progression by retaining monocytes and macrophages within the lesion (6, 17). The transcriptional regulation of both ICAM-1 and IP-10 has shown to involve several transcription factors, including STAT1, IRF1, and nuclear factor (NF)- $\kappa$ B (4, 15, 21, 23, 27). IRF1 expression is upregulated by IFN $\gamma$  in a STAT1-dependent manner and subsequently a major mediator of long-term (and protein-synthesis dependent) IFN $\gamma$  signaling. NF- $\kappa$ B does not participate in IFN $\gamma$  signaling, but like IRF1 (as shown here), plays a direct role in pro-inflammatory pathways activated by LPS, IL-1 $\beta$ , and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ). Our studies in ECs and VSMCs confirmed that IP-10 and ICAM-1 are IFN $\gamma$ -inducible (23, 33) as well as LPS-inducible (5, 8) genes while their expression coincided with STAT1 phosphor-



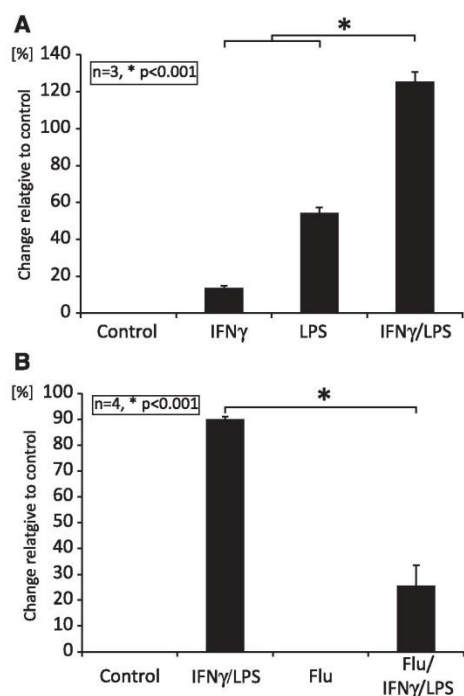


Fig. 5. Increased adhesion of U937 cells to IFN $\gamma$  and LPS-treated HMECs is inhibited by fludarabine. Adhesion of U937 cells was measured to HMECs treated with 10 ng/ml of IFN $\gamma$  for 8 h and/or 1  $\mu$ g/ml of LPS for 4 h in the absence (A) or in the presence of fludarabine (B) (see MATERIALS AND METHODS). Graphs represent mean data from at least three independent experiments  $\pm$  SE. Bars in graphs represent increase in adhesion of U937 cells to HMECs treated with different agents, relative to control sample. Data were tested for significance by one-way ANOVA, followed by post hoc Tukey HSD test. See text for details.

ylation, although in a different way. LPS-induced expression correlated with a protein synthesis-dependent effect for IP-10 and a potential protein synthesis-independent effect for ICAM-1. This suggested a differential role of STAT1 in LPS-mediated transcriptional regulation of IP-10 compared with that of ICAM-1, which was confirmed by experiments including fludarabine.

In case of IP-10, the dramatic increase in gene expression observed in ECs and VSMCs treated with IFN $\gamma$  followed by LPS was absent in TLR4 $^{-/-}$  cells and in the presence of fludarabine, which correlates with a predominant STAT1-dependent mechanism involved in the integration of both signals. Interestingly, the synergism between IFN $\gamma$  and IL1 $\beta$  or TNF- $\alpha$  in mouse embryonic fibroblasts (MEFs) and macrophages was shown to result in increased expression of IP-10 in a STAT1-dependent manner and requiring IRF1 but not NF- $\kappa$ B (21, 26, 27). Since a protein synthesis-dependent mechanism was involved and IRF1 is up-regulated by both IFN $\gamma$  and LPS, it is tempting to speculate that a similar IRF1-mediated mechanism could play a role in IP-10 expression in IFN $\gamma$ -primed vascular cells that are subsequently stimulated by LPS.

In contrast, the increased expression of ICAM-1 RNA in ECs treated with IFN $\gamma$  followed by LPS was weaker compared with IP-10 and only partially affected by fludarabine, implicating additional involvement of a STAT1-independent mechanism. This most likely involves NF- $\kappa$ B, since the protein synthesis-independent character of NF- $\kappa$ B activation by LPS is in agreement with the cycloheximide-independent expression of ICAM-1 under these conditions. IFN $\gamma$ -induced ICAM-1 expression was shown to be STAT1 dependent (33). Indeed the ICAM-1 promoter contains STAT1 and NF- $\kappa$ B binding sites, and maximal transcription requires both signals (4, 15, 22, 31). This suggests that a mechanism involving the cooperation between STAT1 (IFN $\gamma$  and LPS-mediated) and NF- $\kappa$ B (strictly LPS mediated) is responsible for ICAM-1 expression in IFN $\gamma$ -primed vascular cells that are subsequently stimulated by LPS.

Together, these mechanisms could provide an explanation for the differential STAT1-dependent IP-10 and ICAM-1 expression in the presence of both IFN $\gamma$  and TLR signals, relative to signaling with either agonist alone (also summarized in Fig. 6). Nevertheless, it does not exclude the possibility that other molecular mechanisms and other transcription factors may also be involved (25).

Our observation that fludarabine was also able to inhibit IFN $\gamma$ - and LPS-dependent protein expression of IP-10 and ICAM-1 as well as dramatically reduce adhesion of monocytes to ECs in vitro, is in line with a prominent role for both IP-10 and ICAM-1 in this phenomenon. Importantly, fludarabine induced significant reduction of STAT1 phosphorylation and expression in ECs in vitro, whereas it did not affect the expression and activation of STAT3 (data not shown) (9). Thus these data strongly suggest that fludarabine inhibits adhesion of monocytes to ECs by specifically reducing STAT1 activation. Consequently, the signal integration between IFN $\gamma$  and LPS signaling may have important biological significance since both pathways are simultaneously activated during pathogenesis of atherosclerosis. Therefore, in the presence

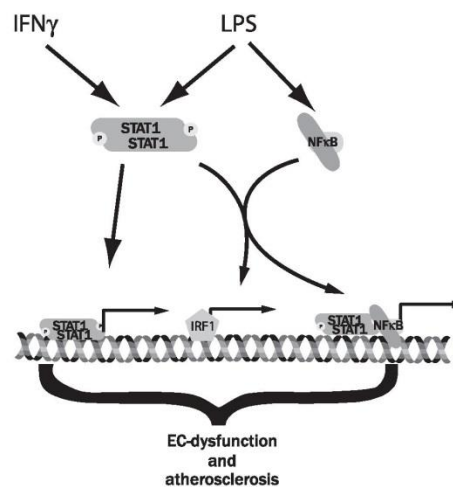


Fig. 6. The central role of STAT1 in signal integration between IFN $\gamma$  and LPS in EC dysfunction and atherosclerosis. Detailed description is given in the text.

of IFN $\gamma$  and LPS (or any other exogenous or endogenous TLR4 ligands), IP-10 and ICAM-1 can be produced in ECs and VSMCs and may in turn function on leukocyte attraction and adhesion and VSMC proliferation and migration, which are important characteristics of EC dysfunction and early triggers of atherosclerosis.

With the established roles of IFN $\gamma$  and TLRs in atherosclerotic pathology, the synergism between IFN $\gamma$  and TLRs in ECs and VSMCs and atheroma-interacting immune cells in response to exogenous and endogenous atherogenic ligands could result in amplification of STAT1, IRF1, and NF- $\kappa$ B-mediated pro-inflammatory responses in the damaged vessel and be a novel mechanism involved in EC dysfunction and the initiation and progression of atherosclerosis (Fig. 6). As such, STAT1 could potentially represent a novel target of therapeutic intervention that has a crucial role in mediating the interplay between damaged vessels and host immunity to control atherosclerosis mediated by IFN $\gamma$  and TLR-directed cross talk.

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#### DISCLOSURES

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# STAT1 as a central mediator of IFN $\gamma$ and TLR4 signal integration in vascular dysfunction

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**Keywords:** atherosclerosis, IFN-gamma, Toll-like receptors, STAT1, IRFs, signal integration

Atherosclerosis is characterized by early endothelial dysfunction and altered vascular smooth muscle cells (VSMCs) contractility. The forming atheroma is a site of excessive production of cytokines and inflammatory ligands by various cell types that mediate inflammation and immune responses. Key factors contributing to early stages of plaque development are IFN $\gamma$  and TLR4. This review provides insight in the differential STAT1-dependent signal integration between IFN $\gamma$  and TLR4 signals in vascular cells and atheroma interacting immune cells. This results in increased leukocyte attraction and adhesion and VSMC proliferation and migration, which are important characteristics of EC dysfunction and early triggers of atherosclerosis.

## Introduction

Atherosclerosis and arteriosclerosis, the morphological correlates of vascular disease, are characterized by early endothelial dysfunction and altered contractility of vascular smooth muscle cells (VSMCs). In 1856 Rudolf Virchow presented a theory that inflammation is the driving force of atherosclerosis. However, scientific proof for this was discovered only 30 years ago.<sup>1</sup> Inflammation participates importantly in host defenses against infectious agents and injury, but it also contributes to the pathophysiology of atherosclerosis. Various factors can injure vascular endothelium leading to the release of numerous inflammatory mediators resulting in recruitment of blood leukocytes, which inflict further inflammatory response. Thus, cells of both innate and adaptive immunity modulate the chronic inflammatory process initiating and acting in the atherosclerotic plaque development.<sup>2</sup> The change in the milieu prompts vascular smooth muscle cells to undergo de-differentiation characterized by loss of contractility, increased cell motility and proliferation.<sup>3</sup> These processes together with buildup of lipids, cholesterol, calcium and cellular debris within the intima of the vessel wall

lead to the formation of advanced atherosclerotic plaque, vascular remodeling and acute and chronic luminal obstruction.<sup>4</sup>

A seminal signal transduction pathway operating at the frontier of innate and adaptive immunity and importantly contributing to inflammation is the JAK-STAT pathway. The involvement of this pathway in atherosclerosis has been long appreciated.<sup>5,6</sup> However, until only recently the attention of the researchers was primarily focused on immune cells. As such it has become clear that for instance in macrophages, dendritic cells as well as lymphocytes, JAK-STAT-mediated signal integration exists between triggers of innate and adaptive immunity, which forms a basic aspect of the host defense against pathogens. Work of more recent nature, including that of our group, uncovered the unique role of STAT1 in cross-talk between the pro-inflammatory activators IFN $\gamma$  and LPS. Moreover, this signal integration takes place not only in immune cells, but also in cells from the vasculature (ECs and VSMCs) and collectively leads to increased inflammation and progression of vascular damage.<sup>4,7</sup> In this review we will summarize the molecular mechanisms of this phenomenon and highlight the biological impact of these findings that could potentially lead to discovery of novel pharmaceutical targets and diagnostic and prognostic assay development.

## Atherosclerosis, Inflammation and Immunity

The function of healthy arterial endothelium is to maintain proper blood flow and provide a physical barrier between blood and surrounding tissue. This is achieved by the ability of endothelial cells to inhibit thrombosis, leukocyte adhesion, VSMC proliferation and to regulate vessel tone. The very first incident in the chain of events leading to the formation of atherosclerotic plaques is endothelial cell injury and the resulting endothelial cell dysfunction (Fig. 1).<sup>8</sup> Although there is still some debate on this, factors inducing EC dysfunction include: pulsatile blood flow and shear stress,<sup>9,10</sup> oxidized LDL particles,<sup>11</sup> pathogens,<sup>12</sup> endogenous damage associated molecules (HSPs),<sup>13</sup> fragments of extracellular matrix<sup>14,15</sup> and pro-inflammatory cytokines released elsewhere in the organism carried by blood. Many of the above mentioned ligands act by activating scavenger receptors and pattern recognition receptors (PRRs), which are key elements of the innate immunity and are expressed on the surface of ECs.<sup>16</sup> In response to cytokines, ECs produce chemokines, pro-inflammatory cytokines and also

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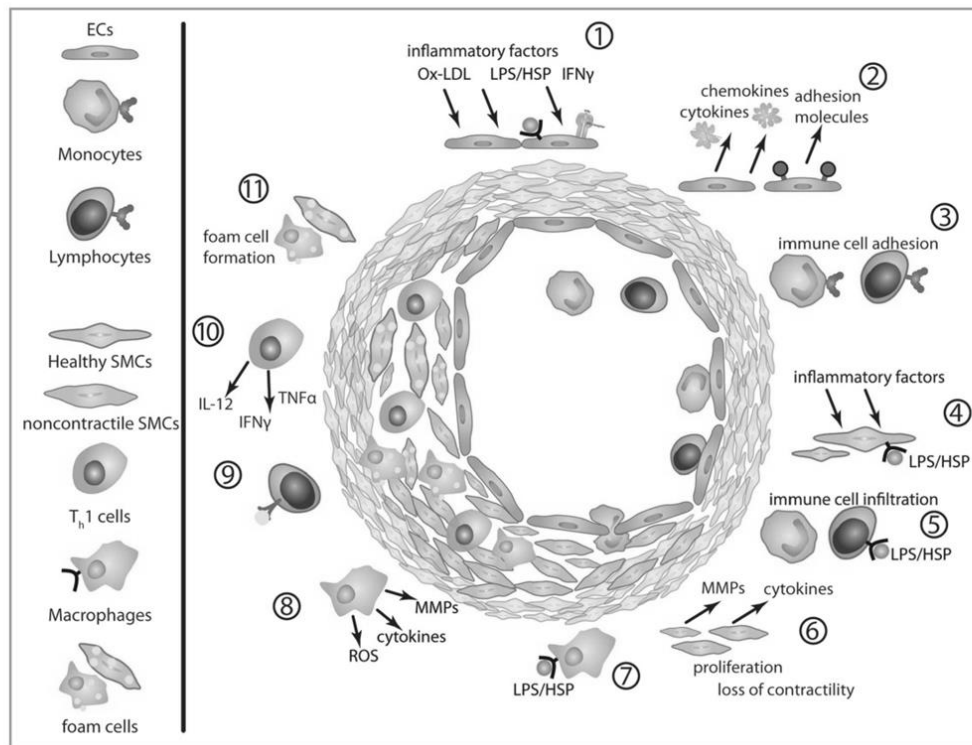


express adhesion molecules. This attracts circulating leukocytes and allows them to adhere to ECs and translocate into the intima. There, monocytes differentiate into macrophages (reviewed by Hoeksema et al.<sup>17</sup>), which phagocytize oxidized LDL and become foam cells, releasing pro-inflammatory cytokines, chemokines and matrix metalloproteinases (MMPs). T lymphocytes differentiate into T helper subsets, including T<sub>H</sub>1, T<sub>H</sub>2, T<sub>H</sub>17 and T<sub>reg</sub> cells. Among the different lymphocyte subsets, T<sub>H</sub>1 cells are considered to be pro-atherogenic, T<sub>reg</sub> atheroprotective and the role of T<sub>H</sub>2 and T<sub>H</sub>17 cells is still debated with contradictory evidence being published (reviewed by Butcher and Galkina).<sup>18</sup> The release of cytokines and MMPs by the activated immune cells and ECs triggers VSMCs to proliferate, migrate and form foam cells resulting in vessel occlusion and neointima formation (Fig. 1). As such, the forming atheroma is a site of excessive production of

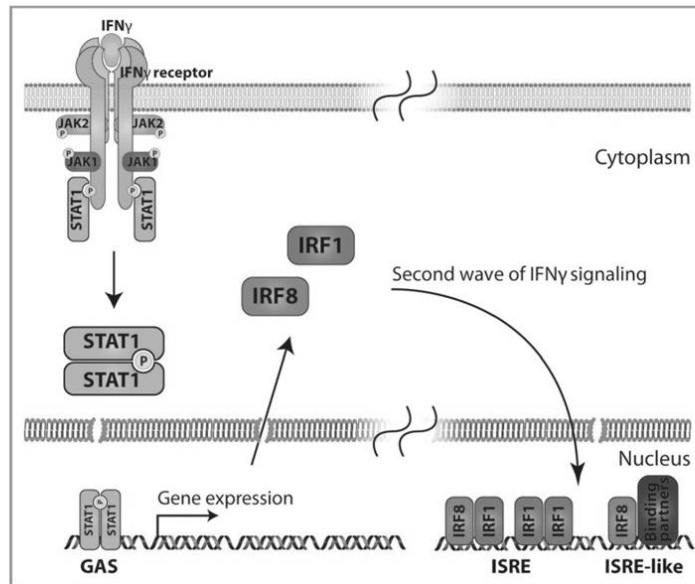
cytokines and other inflammatory ligands by various cell types that mediate inflammation and immune responses, and promote vascular remodeling and tissue damage.

### IFN $\gamma$ in Atherosclerosis

Since its discovery in 1970<sup>19</sup> interferon (IFN) $\gamma$  has been recognized to not only stimulate immune cells and protect against viral infection, but also play a key role in a number of inflammatory diseases, such as atherosclerosis. The innate and adaptive immunity responses are both affected by the pro-inflammatory IFN $\gamma$ , which is produced mainly by T<sub>H</sub>1 cells. IFN $\gamma$  acts by activating macrophages, natural killer cells and B cells, but also vascular cells: smooth muscle and endothelial. The key role of IFN $\gamma$  in atherosclerotic plaque development was



**Figure 1.** Atherosclerotic plaque development. The innate and adaptive immunity co-operate in an inflammatory process that leads to vessel occlusion. (1) Inflammatory triggers induce EC dysfunction by activating the JAK-STAT pathway and PRRs. (2) ECs release inflammatory cytokines, chemokines, adhesion molecules. EC layer permeability increases. (3) Procoagulant surface of ECs promotes leukocyte adhesion and activation. (4) The inflammatory stimuli acts also on SMCs. (5) Leukocytes infiltrate the intima. (6) Activated SMCs produce cytokines and MMPs, which further change the microenvironment. The cells lose contractility and gain motility and proliferate. (7) The infiltrating monocytes transform into macrophages, (8) producing MMPs, further cytokines and ROS. (9) T-cells react to auto-antigens like ox-LDL and differentiate (10) into effector subsets, producing additional cytokines. (11) Macrophages and SMCs phagocytize lipid particles and become foam cells.



**Figure 2.** IFN $\gamma$  signaling. Upon ligation, two IFNGR1 and two IFNGR2 chains create a tetrameric receptor. The cytoplasmic tails are bound by JAK1 and JAK2 kinases respectively. The close proximity allows the kinases to cross-phosphorylate each other. The activated kinases then phosphorylate specific tyrosine residues on the cytoplasmic domain of IFNGR1, creating docking sites for STAT1 transcription factor. Bound STAT1 is phosphorylated, dissociates from the receptor and creates active homodimers. These can translocate to the nucleus, where they bind the GAS element in the promoter region of interferon stimulated genes, which comprises the first, immediate response to IFN $\gamma$ . Among these genes are IRF transcription factors, which act in a delayed manner, to sustain IFN induced response. IRF1 can act as a homodimer binding to the ISRE element. IRF8 cannot bind DNA by itself, and so acts as a heterodimer with IRF1 or other binding partners (e.g., PU.1) activating ISRE or ISRE-like elements.

supported by evidence from mouse models of plaque formation. It was shown that serological neutralization or genetic absence of IFN $\gamma$  significantly reduces atheroma formation.<sup>20,21</sup> In addition, IFN $\gamma$  was found to be highly expressed within atherosclerotic lesions, further proving its critical role in atherogenesis and modeling of cell behavior and cell-cell interactions of all cell types existing in the vessel wall.<sup>22</sup> Until now, most reports on the role of IFN $\gamma$  in atherosclerosis depicted this cytokine as pro-inflammatory with a role in the development and progression of the plaque. IFN $\gamma$  dependent events include activation and differentiation of T-cells, as well as macrophage-mediated release of inflammatory cytokines, specifically TNF $\alpha$  and IL6, and pathological amounts of nitric oxide. In addition to that, IFN $\gamma$  can induce adhesion, cell apoptosis and matrix deposition, all of which resemble endothelial cell dysfunction and were shown to contribute to atherosclerotic lesion development.<sup>22</sup> Lastly, the formation of SMC foam cells and inhibition of SMC proliferation is promoted by IFN $\gamma$  both in vitro and in vivo.<sup>23</sup>

### IFN $\gamma$ Signaling Pathways

IFN $\gamma$  is the sole member of the type II interferon class and as such mediates most of the cell responses through the now well-known

JAK-STAT pathway (Fig. 2). Binding of homodimeric IFN $\gamma$  leads to the activation of type II interferon receptor. The active receptor complex consists of two IFNGR1 chains and two IFNGR2 chains, which on the cytoplasmic side are associated with JAK1 and JAK2 kinases respectively. Bringing these kinases in close proximity allows them to cross-phosphorylate each other on specific tyrosine residues. Active JAK kinases can in turn phosphorylate the cytoplasmic domains of the IFNGR1 creating docking sites for the signal transducer and activator of transcription (STAT)-1 protein. STAT1 is a member of a family of transcription factors comprising in total seven proteins.<sup>24-26</sup> STAT proteins share structural homology: N-terminal domain (ca. 140 a.a.), coiled-coil domain (ca. 180 a.a.), DNA binding domain (ca. 170 a.a.), linking domain (ca. 90 a.a.), SH2 domain (ca. 140 a.a.) and transcriptional activation domain. The N-terminal domain is involved in dimer complex formation and methylation, the coiled-coil region interacts with other transcription factors and the SH2 domain interacts with phosphorylated tyrosine motifs. Receptor bound STAT1 is phosphorylated, dissociates from the receptor and creates active homodimers that translocate to the nucleus where, by binding to interferon gamma activated sequence (GAS), they activate transcription.



STAT1-target genes include chemokines (IP-10 and CCL5), adhesion molecules (ICAM-1) and transcription factors (IRF1 and IRF8).<sup>24,25,27</sup>

### IFN $\gamma$ and IRFs

While the STAT1 homodimer is the primary and best characterized route to IFN $\gamma$ -induced transcription, IFN $\gamma$  additionally activates transcription factors of the interferon regulatory factor (IRF) family (Fig. 2). Currently nine members of this family have been identified in mammals (named IRF 1 to 9). All IRFs share a highly conserved N-terminal DNA-binding domain, which is ultrastructurally characterized by a helix-loop-helix motif with a signature tryptophan pentad. IRFs were identified as transcription factors that specifically bind to a highly conserved consensus site in the promoter region of type I IFN and interferon-inducible genes, named IFN-stimulated response element (ISRE).<sup>24,28</sup> In this way IRFs were found to regulate the expression of many genes that play a pivotal role in a host of cellular functions such as proliferation, apoptosis, cell cycle regulation and regulation of innate and adaptive immune defense.<sup>28</sup>

In the context of IFN $\gamma$  crucial role play IRF1 and IRF8, particularly by amplifying expression of IFN $\gamma$ -responsive genes initiated by STAT1 (Fig. 2).<sup>26</sup> In contrast to STAT1 and IRF1, which are ubiquitously expressed, IRF8 expression is thought to be restricted to lymphoid-cell lineages such as B, T and dendritic cells and macrophages. Accordingly, IRF8 was shown to take part in unique subsets of ISRE-mediated (named EIRE and EICE) but also in GAS-mediated transcription in co-operation with other IRFs (IRF1) or other transcription factors including PU.1 (also immune cell restricted) to drive the differentiation of these lineages. Thus, IRF8 may in part account for "immune cell-specific" STAT1-dependent functions of IFN $\gamma$ .<sup>29</sup> Interestingly, recently we obtained evidence for the first time that IRF-8 is highly expressed in EC and VSMC after IFN $\gamma$  treatment<sup>30</sup> (Chmielewski et al., manuscript in preparation), suggesting that it could also regulate "vasculo-specific" STAT1-dependent functions of IFN $\gamma$ . The role of IRF-8 in EC and VSMC (dys)function has not been studied but, it is tempting to speculate that IRF-8 specifically regulates STAT1-dependent IFN $\gamma$ -directed transcriptional responses in cell types involved in vascular dysfunction. In addition, IRF8 could potentially mediate IFN $\gamma$ -directed cross-talk between EC, VSMC and atheroma-interacting immune cells.

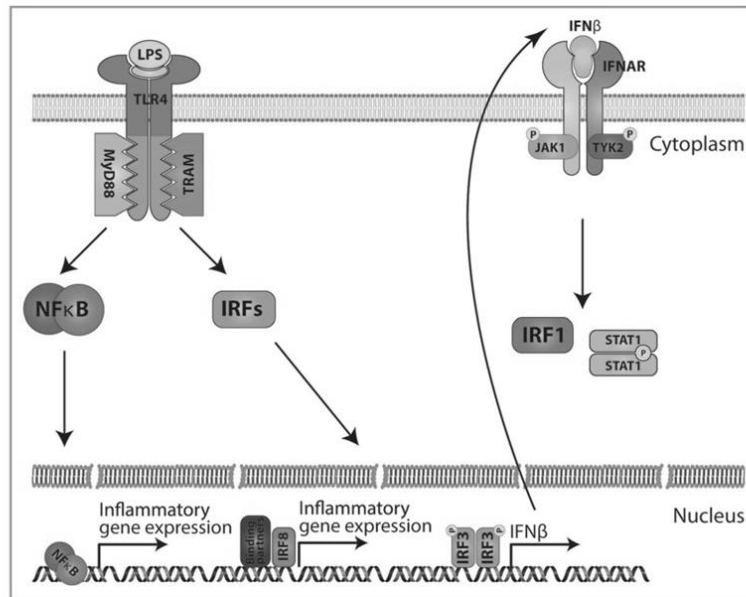
### TLR4 Signaling

Toll-like receptors (TLRs) are a more recent discovery than interferons. The name of the family comes from the *Drosophila* Toll receptor, which was originally recognized to have a role in embryonic development.<sup>31</sup> It was only in 1996 that its fundamental contribution to the fly's innate immune response to fungi was revealed by a group of J. Hoffmann.<sup>32</sup> The first human TLR was discovered in 1994,<sup>33</sup> but its subsequent role in defense against pathogens was uncovered in 1997.<sup>34,35</sup> Data obtained by many research groups since then showed that TLRs

are important regulators of the innate immune system. To date, there have been at least 13 TLRs discovered in mammals, 10 of which are expressed in human cells.<sup>36</sup> TLRs are responsible for detection and recognition of multiple exogenous pathogen associated molecular patterns (PAMPs) and endogenous damage associated molecular patterns (DAMPs). This function places them at the frontline of the host cell response to infection, inflammation and injury. Exogenous molecules recognized by TLRs include: bacterial lipoproteins and lipoteichoic acid (TLR2), double stranded RNA (TLR3) and lipopolysaccharide (TLR4). TLR2 and TLR4 can also be ligated by stress or injury-induced altered host-derived ("self") structures, which include heat shock proteins and products of extracellular matrix degradation.<sup>36</sup> Depending on the receptor and the nature of the ligand, the active receptor complex may form either a dimer or a monomer with the cytoplasmic domain of the receptor acting as a scaffold for multiple adaptor proteins which relay the signal downstream. This cytoplasmic domain, namely Toll/IL-1 resistance (TIR) domain, is present on all TLRs and their adapters and allows interactions between them.<sup>36</sup> The adaptor protein myeloid differentiation protein-88 (MyD88) is involved in signaling of most TLRs, with the exception of TLR3.<sup>37</sup> TLR4 (Fig. 3) was shown to utilize all four described TIR-containing adapters: MyD88 and MAL (MyD88-adaptor-like; also known as TIRAP) seem to act in pair to activate the MyD88-dependent pathway resulting in NF $\kappa$ B activation, whereas TIR domain-containing adaptor protein inducing IFN $\beta$  (TRIF; also known as TICAM1) and TRIF-related adaptor molecule (TRAM) in pair activate the interferon pathway.<sup>38</sup> Together this leads to the induction of various target genes that include type I IFNs, chemokines and cell surface molecules. Since a pro-arteriosclerotic effect has been demonstrated for most of these, TLRs as the main endogenous contributor have also been investigated for their potential role in the development of arteriosclerosis.

### TLR-Mediated Immune Responses in the Vessel

In particular, TLR4 is expressed in both human and mouse atherosclerotic lesions. Expression has mainly been located to endothelial cells and macrophages within the lesion. Also, patients with acute coronary syndromes or coronary arteriosclerotic lesions have an increased TLR4 expression on circulating monocytes as compared with control patients.<sup>39</sup> Finally, there is evidence that increased TLR expression correlates with endothelial dysfunction in cardiac transplant recipients.<sup>40</sup> Mice deficient in TLR4 have reduced atherosclerosis which establishes that Toll-like receptor dependent pathways contribute to disease development.<sup>41</sup> Similarly, TLR4 has been implicated in vascular inflammation in an angiotensin II directed mouse model of vascular dysfunction.<sup>42</sup> Moreover, TLR4<sup>-/-</sup> mice are protected against obesity.<sup>43</sup> Further evidence that TLR signaling is important in ischemia-reperfusion injury comes from myocardial ischemia models in which TLR4 signaling is important for infarct size and subsequent left ventricular dysfunction.<sup>44</sup> Thus, experimental and clinical evidence exists that TLR4 signaling at the very least participates in vascular damage.



**Figure 3.** TLR4 signaling (simplified). LPS binds the homodimeric TLR4 receptor with the help of MD2 molecule. Ligation of the receptor allows the cytoplasmic TIR domain to interact with accessory molecules. Generally, there are two pathways. One, through MyD88 leads to activation of the NFκB transcription factor, which induces expression of inflammatory cytokines. The second pathway, through TRAM, activates IRFs. IRF8 interacts with other transcription factors leading to inflammatory gene expression. IRF3 is phosphorylated, homodimerizes and induces IFNβ production, which in an auto- and paracrine way can stimulate cells to induce IRF1 and STAT1 in the JAK-STAT dependent manner.

### STAT1 and IRF8 in TLR Signaling

TLR signaling leads to the induction of various target genes that include those encoding type I IFNs, pro-inflammatory cytokines, chemokines and cell surface molecules. Some of these genes are regulated secondary to LPS-induced IFNβ, which after secretion binds to the type I IFN receptor to activate gene expression (including IP-10, MCP-1, CCL5, ISG15 and iNOS) in a STAT1-dependent manner (Fig. 3). As such, STAT1 has been identified as an important mediator in the biological response to different TLRs, including TLR4.

TLRs have also been shown to utilize the IRF family. Specifically, IRF1, IRF3, IRF5, IRF7 and IRF8 were shown to contribute to TLR-mediated signaling.<sup>28</sup> IRF1,<sup>45</sup> IRF5<sup>46</sup> and IRF7<sup>47</sup> directly interact with MyD88 in TLR9 signaling. This interaction allows for their activation and subsequent translocation to the nucleus, where they can induce gene expression. IRF3 is constitutively expressed and upon TLR4 activation is phosphorylated in the Myd88 independent pathway leading to IFNβ expression, which in turn can induce IRF1 expression.<sup>48</sup> IRF8 is at several levels connected to the TLRs. Independent of TLRs, IRF8 increases TLR gene expression in B-cells and myeloma cells.<sup>49</sup> As a TLR signaling component, IRF8 interacts

with TNF-receptor associated factor (TRAF) 6, a MyD88 recruited ubiquitin ligase, and regulates the production of type I IFNs and other inflammatory mediators. In this way, unmethylated CpG via TLR9 induces activation of IRF8 in dendritic cells that is mediated by NFκB. Consequently, IRF8-deficient mice reveal a signaling defect of TLR9-mediated induction of TNFα and interleukin-6.<sup>50</sup> In dendritic cells IRF8 also facilitates TLR2 and TLR4 mediated induction of interleukins, NO synthase and TNFα that involves activation of several kinases like ERK, JNK and MAP kinase.<sup>51</sup> IRF8-deficient mice are highly susceptible to several pathogens, including *Listeria monocytogenes*<sup>52</sup> and lymphocytic choriomeningitis virus,<sup>53</sup> due to defects in both innate and adaptive immunity. In addition, macrophages from IRF8<sup>-/-</sup> mice produce diminished levels of TNFα, IL1β and IL12p70 in response to LPS.

### STAT1- and IRF8-Mediated Crosstalk between IFNγ and LPS

The synergy between IFNγ and TLR has been implicated in the host defense against pathogens. IFNγ produced by T cells and other cells is considered to enhance TLR signaling in DCs and macrophages for the efficient induction of inflammatory

mediators to eliminate pathogens.<sup>54,55</sup> Lately, evidence has been provided on the mechanistic insights of this cross-talk between IFN $\gamma$  and TLR signaling pathways, with STAT1 being a critical mediator.<sup>7,56,57</sup> In general it is believed that the transactivation ability of the macrophage STAT1 pool is super-activated upon stimulation with both IFN $\gamma$  and LPS, relative to either agonist alone.<sup>7,54</sup> The STAT1-targets IRF1 and IRF8 have also been shown to contribute to the signal integration between IFN $\gamma$  and LPS. IRF1 is a major mediator of IFN $\gamma$  signaling, and is regulated by both IFN $\gamma$  and TLR agonists as a consequence of STAT1 and NF $\kappa$ B elements in the IRF1 promoter that mediate transcriptional induction.<sup>58-61</sup> Zhao et al.<sup>51</sup> reported that in macrophages IRF8 was upregulated in response to either IFN $\gamma$  or LPS, and was super-induced by IFN $\gamma$  and LPS co-administration. Correspondingly, IFN $\gamma$  and LPS synergistically induced the expression of pro-inflammatory factors, including IL-1, IL-6, IL-12, NO and TNF- $\alpha$ , in an IRF8-dependent manner. Comparable synergism was observed between IFN $\gamma$  and peptidoglycan (PGN; a TLR2 ligand) and poly (I:C) (a TLR3 ligand) in the induction of IL-12 promoter activity. Also in macrophages, IRF8 has been linked to the IFN $\gamma$  and LPS-mediated transcriptional activation of CCL5 (RANTES),<sup>62</sup> a known chemokine actively involved in leukocyte recruitment to the injured artery during vascular remodeling. These findings suggest that in immune cells STAT1 and IRF8 are unique points of convergence for the antimicrobial synergism between IFN $\gamma$  and TLRs.

Our recent observations<sup>7</sup> suggest that also in ECs and VSMCs STAT1 orchestrates a platform for cross-talk between IFN $\gamma$  and LPS. Moreover, increased production of STAT1 protein in these cells, strictly dependent on IFN $\gamma$  provides a potential mechanism resulting in augmented STAT1 phosphorylation when both IFN $\gamma$  and LPS are present. This coincided with increased expression of the chemokine IP-10 and the adhesion molecule ICAM-1 as well as adhesion of U937 leukemia cells to ECs, in a STAT1- and TLR4-dependent manner.<sup>7</sup> Interestingly, under the same conditions we also observed a significant increase in IRF8 gene expression as compared with both factors alone. More importantly, this correlated with a dramatic amplification of CCL5 (RANTES) gene and protein expression in a STAT1 as well as IRF8-dependent fashion. (Chmielewski et al., manuscript in preparation)

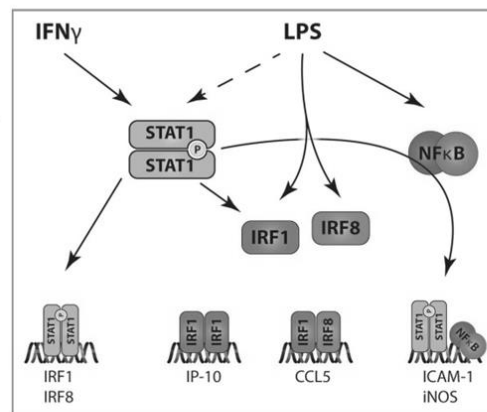
In addition to its known immune cell functions, this uncovers a novel role of IRF8 in ECs as well as VSMCs, facilitating signaling events initiated by both IFN $\gamma$  and LPS, thus providing a platform for IRF8-dependent crosstalk between the two pathways and leading to increased expression of pro-inflammatory mediators.

#### Multiple Signal Convergence of STAT1 in Vascular Dysfunction

Integration of IFN $\gamma$  and TLR signaling pathways occurs through synergy between TLR- and IFN $\gamma$ -induced transcription factors, which is likely to be a global mechanism to allow the integration of multiple input signals for synergistic, coordinated regulation of

gene expression. IRF1, IP-10, ICAM-1 and iNOS genes contain both STAT1 and NF $\kappa$ B binding sites in their promoter.<sup>59,60,63-68</sup> For example, IFN $\gamma$  signaling induced iNOS mRNA via STAT1, but induction was maximal only when the iNOS promoter NF $\kappa$ B sites were occupied following TLR ligation.<sup>64,68,69</sup>

More detailed investigation of STAT1-dependent transcriptional synergism between IFN $\gamma$  and LPS in cells from the vasculature predicts the existence of different regulatory mechanisms through the additional involvement of IRF1 and IRF8 (Fig. 4). Our studies in ECs and VSMCs confirmed that IP-10 and ICAM-1 are IFN $\gamma$ <sup>70,71</sup> as well as LPS-inducible<sup>72,73</sup> genes while their expression coincided with STAT1 phosphorylation, although in a different way. In case of IP-10, the dramatic increase in gene expression observed in ECs and VSMCs treated with IFN $\gamma$  followed by LPS correlated with a predominant STAT1-dependent, but IRF8-independent (Chmielewski et al., manuscript in preparation) mechanism involved in the integration of both signals. Interestingly, the synergism between IFN $\gamma$  and IL1 $\beta$  or TNF $\alpha$  in mouse embryonic fibroblasts and macrophages was shown to result in increased expression of IP-10 in a STAT1-dependent manner and requiring IRF1, but not NF $\kappa$ B.<sup>66,74,75</sup> Since a protein synthesis-dependent mechanism was involved and IRF1 is upregulated by both IFN $\gamma$  and LPS, it is tempting to speculate that a similar IRF1-mediated mechanism could play a role in IP-10 expression in IFN $\gamma$ -primed vascular cells that are subsequently stimulated by LPS.<sup>7</sup>



**Figure 4.** IFN $\gamma$  and LPS signaling integration in vascular cells. IFN $\gamma$  and LPS use the same transcription factor (STAT1) to elicit cell response. This cross-talk causes amplification of STAT1 activation. This pro-inflammatory factor can induce secondary transcription factor genes by itself (IRF1 and IRF8) and also interact with LPS-dependent NF $\kappa$ B leading to elevated expression of ICAM-1 and iNOS. The second wave of signaling includes IRF1 and IRF8, which are both induced by STAT1 and also directly by LPS. IRF1 can act as a homodimer to induce expression of IP-10 and also can interact with IRF8 to induce CCL5. This creates a multi-layer integration of signaling between IFN $\gamma$  and LPS which leads to increased inflammation and vascular dysfunction.

In contrast, the increased expression of ICAM-1 RNA in ECs treated with IFN $\gamma$  followed by LPS was weaker as compared with IP-10 and most likely involved an NF $\kappa$ B-dependent mechanism.<sup>7</sup> IFN $\gamma$ -induced ICAM-1 expression was shown to be STAT1-dependent.<sup>71</sup> Similar to the iNOS gene, the ICAM-1 promoter contains STAT1 and NF $\kappa$ B binding sites and maximal transcription requires both signals.<sup>59,63,76</sup> This suggests that a mechanism involving the cooperation between STAT1 (IFN $\gamma$  and LPS-mediated) and NF $\kappa$ B (strictly LPS-mediated) is responsible for ICAM-1 expression in IFN $\gamma$ -primed vascular cells that are subsequently stimulated by LPS.

The transcriptional regulation of the CCL5 gene in macrophages in response to IFN $\gamma$  and LPS uncovered a novel role of IRF8 and required both the IRF1 and NF $\kappa$ B binding sites.<sup>62</sup> Indeed, IRF8 complexed with IRF1 at the ISRE was responsible for the IFN $\gamma$  signal, while IRF8 interacting with NF $\kappa$ B and PU.1 at the NF $\kappa$ B site in the IFN $\gamma$  and LPS response.<sup>62</sup> PU.1 is a member of the large Ets family and is an important regulator of myeloid and lymphoid cell differentiation.<sup>77</sup> Since PU.1 expression is restricted to immune cells and IRF1 and IRF8 are upregulated by both IFN $\gamma$  and LPS, the IRF8-dependent expression of CCL5 in IFN $\gamma$ -primed vascular cells that are subsequently stimulated by LPS (Chmielewski et al., manuscript in preparation) is highly likely to involve an IRF1/IRF8-mediated mechanism and not an IRF8/NF $\kappa$ B.

### Future Perspectives

Together with the established roles of IFN $\gamma$  and TLRs in atherosclerotic pathology, the synergism between IFN $\gamma$  and TLRs in ECs and VSMCs and atheroma-interacting immune cells in response to exogenous and endogenous atherogenic ligands could result in amplification of STAT1-mediated pro-inflammatory responses in the damaged vessel. In co-operation, the mechanisms as proposed in Figure 4 could offer an explanation for the differential STAT1-dependent signal integration between IFN $\gamma$  and TLR4 signals in vascular cells, with the novel role of IRF8 providing an additional layer to the overall complexity. As a consequence, in the presence of IFN $\gamma$  and LPS (or any other exogenous or endogenous TLR4 ligands), pro-inflammatory mediators like IP-10, CCL5 and ICAM-1 can be over-produced

in ECs and VSMCs and may in turn function on leukocyte attraction and adhesion and VSMC proliferation and migration,<sup>78-81</sup> which are important characteristics of EC dysfunction and early triggers of atherosclerosis.

**Translational implications.** Despite the tremendous progress made in atherosclerosis management, it is still a common health problem and a major financial load on healthcare system. Until now, therapies focused on stabilizing patients with moderate and advanced pathologies, partially because clinicians are lacking diagnostic assays able to detect early changes and partially because of the lack of information about the molecular basis of early plaque development. Novel experimental background summarized in this review brings promise of new intervention and diagnostic tools that could act in the early stages of atherosclerotic plaque development.

STAT1 represents an interesting novel target of therapeutic intervention that has a crucial role in mediating the interplay between damaged vessels and host immunity to control atherosclerosis mediated by IFN $\gamma$  and TLR-directed crosstalk. With its immune cell specificity and novel vascular specific functions, we uncovered IRF8 as an attractive novel therapeutic target that could provide a way to control early plaque formation in a cell type specific manner and with greater specificity.

Finally, crosstalk between IFN $\gamma$  and TLR4 relying on STAT1 and IRF8 results in amplification of expression of inflammatory mediators, such as IP-10, ICAM-1 and CCL5. These mediators could potentially be quantified in the serum of patients and used as a measure of disease initiation and progression. Although the concept of serum markers of atherosclerosis has been pursued already for some time without much success, the new experimental findings summarized in this review show new promise that such specific markers could be selected and used to monitor and diagnose subclinical atherosclerotic changes.

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1 STAT1-dependent signal integration between IFN $\gamma$  and TLR4 in vascular cells reflect pro-  
2 atherogenic responses in human atherosclerosis

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18 **Abstract**

19       Signal integration between IFN $\gamma$  and TLRs in immune cells has been associated with the  
20 host defense against pathogens and injury, with a predominant role of STAT1. We hypothesize  
21 that STAT1-dependent transcriptional changes in vascular cells involved in cross-talk between  
22 IFN $\gamma$  and TLR4, reflect pro-atherogenic responses in human atherosclerosis.

23       Genome-wide investigation identified a set of STAT1-dependent genes that were  
24 synergistically affected by interactions between IFN $\gamma$  and TLR4 in VSMCs. These included the  
25 chemokines Cxcl9, Ccl12, Ccl8, Ccr12, Cxcl10 and Ccl5, adhesion molecules Cd40, Cd74, and  
26 antiviral and antibacterial genes Rsad2, Mx1, Oasl1, Gbp5, Nos2, Batf2 and Tnfrsf11a. Among  
27 the amplified genes was also Irf8, of which Ccl5 was subsequently identified as a new pro-  
28 inflammatory target in VSMCs and ECs. Promoter analysis predicted transcriptional cooperation  
29 between STAT1, IRF1, IRF8 and NF $\kappa$ B, with the novel role of IRF8 providing an additional  
30 layer to the overall complexity. The synergistic interactions between IFN $\gamma$  and TLR4 also  
31 resulted in increased T-cell migration and impaired aortic contractility in a STAT1-dependent  
32 manner. Expression of the chemokines CXCL9 and CXCL10 correlated with STAT1  
33 phosphorylation in vascular cells in plaques from human carotid arteries. Moreover, using data  
34 mining of human plaque transcriptomes, expression of a selection of these STAT1-dependent  
35 pro-atherogenic genes was found to be increased in coronary artery disease (CAD) and carotid  
36 atherosclerosis.

37       Our study provides evidence to suggest that in ECs and VSMCs STAT1 orchestrates a  
38 platform for cross-talk between IFN $\gamma$  and TLR4, and identifies a STAT1-dependent gene  
39 signature that reflects a pro-atherogenic state in human atherosclerosis.



40 **Introduction**

41 Inflammation participates importantly in host defenses against infectious agents and  
42 injury, but it also contributes to the pathophysiology of many diseases including atherosclerosis.  
43 Atherosclerosis is characterized by early endothelial cell (EC) dysfunction and altered  
44 contractility of vascular smooth muscle cells (VSMCs) [1]. Recruitment of blood leukocytes to  
45 the injured vascular endothelium characterizes the initiation and progression of atherosclerosis  
46 and involves many inflammatory mediators, modulated by cells of both innate and adaptive  
47 immunity [2].

48 The pro-inflammatory cytokine interferon (IFN)- $\gamma$ , derived from T-cells, is vital for both  
49 innate and adaptive immunity and is also expressed at high levels in atherosclerotic lesions.  
50 Evidence that IFN $\gamma$  is necessary and sufficient to cause vascular remodeling is supported by  
51 mouse models of atheroma formation, as the serological neutralization or genetic absence of  
52 IFN $\gamma$  markedly reduces the extent of atherosclerosis [3,4,5,6]. The signal transduction pathway  
53 initiated by binding of IFN $\gamma$  to its receptor leads to intracellular phosphorylation of signal  
54 transducer and activator of transcription (STAT)1. Subsequently, STAT1 homo dimerizes and  
55 translocates into the nucleus where it binds to IFN $\gamma$ -activated sequences (GAS elements) in the  
56 promoters of IFN $\gamma$ -inducible genes or at other sites by further interaction with other transcription  
57 factors, [7] including members of the Interferon Regulatory Factor (IRF) family [8,9]. Thus,  
58 STAT1 plays a major role in mediating immune and pro-inflammatory responses. As such, IFN $\gamma$   
59 is considered to participate in promoting atherogenic responses through STAT1-mediated  
60 “damaging” signals, regulating the functions and properties of all cell types present in the vessel  
61 wall. Indeed, Agrawal et al. revealed that Stat1 positively influences lesion formation in  
62 experimental atherosclerosis in vivo and is required for optimal progression of foam cell  
63 formation in macrophages in vitro and in vivo [10]. However, the specific role for Stat1 in human  
64 atherosclerosis has not been previously reported.

65 STAT1 has also been identified as an important mediator in the biological response to  
66 different Toll like receptors (TLRs), which are innate immune pattern recognition receptors  
67 (PRR) expressed on a variety of cells, and initiate and sustain the inflammatory response in  
68 atherosclerosis [11]. Activation of TLR4 through lipopolysaccharide (LPS), which is mediated  
69 by both NF $\kappa$ B and IRF3, leads to the induction of various target genes including type I IFNs,  
70 pro-inflammatory cytokines, chemokines and cell surface molecules [12]. Some of these genes

71 are regulated secondary to LPS-induced IFN $\beta$ , which after secretion binds to the type I IFN  
72 receptor to activate gene expression in a STAT1-dependent manner [7]. Cross-talk between IFN $\gamma$   
73 and TLRs has been associated with the host defense against pathogens and injury. IFN $\gamma$   
74 produced by T-cells and other cells is considered to enhance TLR signaling in dendritic cells and  
75 macrophages for the efficient induction of inflammatory mediators to eliminate pathogens  
76 [13,14]. STAT1 has been identified as a critical mediator in this cross-talk between IFN $\gamma$  and  
77 TLR signaling pathways [15,16]. Consequently, the cooperation of STAT1 with other  
78 transcription factors, including IRFs and NF $\kappa$ B, coordinate the antimicrobial and inflammatory  
79 synergism between IFN $\gamma$  and TLRs in immune cells. Recently, we showed that also in ECs and  
80 VSMCs cross-talk between IFN $\gamma$  and TLR4 resulted in augmented STAT1 phosphorylation and  
81 increased expression of the chemokine CXCL10 and the adhesion molecule ICAM-1 as well as  
82 adhesion of U937 leukemia cells to ECs, in a STAT1- and TLR4-dependent manner [17]. We  
83 hypothesize that STAT1-dependent transcriptional changes in vascular cells involved in cross-  
84 talk between IFN $\gamma$  and TLR4, reflect pro-atherogenic responses in human atherosclerosis.

85 Our study indeed provides evidence that in ECs and VSMCs STAT1 coordinates a  
86 platform for cross-talk between IFN $\gamma$  and TLR4, and identifies a STAT1-dependent gene  
87 signature that reflects a pro-atherogenic state in coronary artery disease (CAD) and carotid  
88 atherosclerosis.

89

90 **Material and Methods**

91 **Cell culture experiments**

92 This investigation conforms with the principles of the NIH Guide for the Care and Use of  
93 Laboratory Animals (NIH Publication, 8th Edition, 2011) and the German Law on the Protection  
94 of Animals was followed. Researchers in charge of the experiment, at Klinikum rechts der Isar  
95 were authorized to breed, house, and sacrifice animals. WT mice (strain background C57BL/6)  
96 were obtained from Charles River Laboratories. STAT1<sup>-/-</sup> and IRF8<sup>-/-</sup> mice (both C57BL/6  
97 background) were kindly provided by Thomas Decker and Carol Stocking, respectively [18].  
98 Before any manipulations, animals were euthanized by cervical dislocation under isoflurane  
99 anesthesia. Primary murine Vascular Smooth Muscle cells (VSMCs) were isolated from  
100 C57BL/6 or STAT1<sup>-/-</sup> or IRF8<sup>-/-</sup> aortas by enzymatic digestion [19]. Human Microvascular  
101 Endothelial Cells (ECs) [20] obtained from Centers for disease control and prevention that were  
102 used in current study, were cultivated in MCDB-131 (Life Technologies) medium containing  
103 10% FBS (PAA), 100U/ml penicillin, 100µg/ml streptomycin, 0.01µg/ml EGF, 0.05µM  
104 hydrocortisone (Sigma), 2mM L-glutamine (PAA). On the day before the experiment for both  
105 cell types full medium was exchanged into medium containing 2% serum. Afterwards cells were  
106 treated with 10ng/ml of IFN $\gamma$  (Life Technologies, PMC4031) and/or 1µg/ml of LPS (Sigma,  
107 L4391).

108

109 **RNA isolation and real-time PCR**

110 Total RNA was isolated from VSMCs and ECs using RNeasy Mini Kit (Qiagen, 74104)  
111 together with DNase digestion step according to the manufacture's protocol. Isolated aortas  
112 were cleaned from perivascular fat and incubated as depicted in Fig 1. After stimulation aortas  
113 were snap frozen on liquid nitrogen, ground up with a pestle and resuspended in 1ml of Trizol.  
114 Total RNA was isolated using Trizol method followed by PureLink RNA kit (Life Technologies,  
115 12183018A ). Complementary DNA was synthesized using iScript cDNA Synthesis Kit  
116 (BioRad, 170-881), according to manufacturer's protocol. Quantitative reverse transcriptase PCR  
117 (qRT-PCR) was performed using SSoFast Evagreen (MyiQ ICycler, Bio-Rad, 172-5201).  
118 Forward and reverse primers are depicted in Suppl Table S4. The 2<sup>-ddCt</sup> method was applied for  
119 quantification [21]. Fold change in the target gene were normalized to GAPDH and relative to

120 the mean expression at untreated sample. The results are expressed as fold of control from at  
121 least 3 independent assays.

122

### 123 **Microarray analysis**

124 VSMCs from WT and STAT1<sup>-/-</sup> were treated as described in Fig. 1. RNA from control and treated  
125 samples was isolated and labeled according to Illumina® TotalPrep™ RNA Amplification Kit  
126 (Life Technologies, AMIL1791). Standard Illumina Expression BeadChip MouseRef-8v2  
127 (Illumina) hybridization protocol was used to obtain the raw data. Chips were scanned using  
128 HiScanSQ system. The complete data of the Illumina Expression BeadChip analysis can be found  
129 at the NCBI GEO, with the accession number GSE49519. The average signals from 3  
130 independent biological experiments were taken for statistical testing. Genes from treated samples  
131 with detection p-value <0.05 were selected for background subtraction and quantile  
132 normalization. Up-regulated genes were considered with p-value <0.05 and at least 2-fold  
133 difference. Genes which expression after co-treatment reached higher level than additive  
134 expression after IFN $\gamma$  or LPS were considered as amplified. Regulated genes in WT cells which  
135 expression was lowered at least by 50% or fold induction was smaller than 2 in STAT1<sup>-/-</sup> was  
136 considered as a STAT1 target. For comparison of up-regulated genes Venn diagram tool was  
137 used (<http://bioinfogp.cnb.csic.es/tools/venny/index.html>) [22]. Gene names from data sets were  
138 used for identifying overlapping genes. Promoters for amplified STAT1 dependent genes were  
139 screened using GENOMATIX software (<http://www.genomatix.de/>) [23]. The promoter regions  
140 from -1000 to +100bp were searched for binding sites (V\$IRF1.01 V\$ISGF3G.01 V\$ISRE.01  
141 V\$ISRE.02V\$CREL.01 V\$NFKAPPAB.01 V\$NFKAPPAB.02 V\$NFKAPPAB65.01  
142 V\$STAT.01 V\$STAT1.01 V\$STAT1.02) or models with core similarity at least 0.85.  
143 Enrichment in gene ontology processes categories was performed using Gorilla software  
144 (<http://cbl-gorilla.cs.technion.ac.il/>) [24]. P-value of 10<sup>-3</sup> was used as a threshold and Illumina  
145 gene lists from HumanHT-12 v4 or MouseRef-8 v2 were taken as a background model. Next, all  
146 the statistically significant and enriched gene ontology categories were analyzed by Revigo  
147 software (<http://revigo.irb.hr/>) [25]. To remove redundant GO terms the allowed similarity value  
148 of 0.5 was used.

149

150 **In silico gene expression analysis.**

151 Human atherosclerotic plaque datasets were downloaded from NCBI Gene Expression Omnibus  
152 repository. Carotid dataset (accession no. GSE21545 [26] contained 223 microarrays (124  
153 samples were used for the analysis) and coronary dataset (accession no. GSE40231 [27]  
154 contained 278 samples (80 arrays were used for the analysis)). As GSE21545 did not contain any  
155 healthy artery controls, these samples were compared against controls from GSE40231. For this  
156 purpose, batch effects between the combined datasets were removed using ComBat tool, a  
157 widely used method for removing variations between batches of arrays [28]. In both cases the  
158 authors isolated RNA from whole plaques obtained from patients during surgery.

159 Raw .cel files downloaded from GEO were normalized using RMA algorithm, signals were log-  
160 transformed and probes were combined to genes using “Combine probes to genes” tool (Chipster  
161 software [29]). Fold change and corresponding p-values were calculated using “calculate fold  
162 change” tool (Chipster [29]). Genes up-regulated at least 1.5 times in both datasets were  
163 compared with a list of 30 genes amplified by IFN $\gamma$  and LPS treatment in VSMCs.

164 The list of STAT1 target genes up-regulated by IFN $\gamma$  and LPS in murine VSMCs was used as the  
165 starting point for promoter analysis. First, that list was fed to pSCAN online promoter analysis  
166 tool in order to look for GAS, interferon stimulated response element ISRE (recognized by IRFs)  
167 and NF $\kappa$ B binding sites. The software was set to analyse 950bp upstream and 50bp downstream  
168 of the transcription start site. PSCAN produced a list of over-represented transcription factor  
169 binding sites together with occurrences of each site and a matrix similarity score. Occurrences  
170 having the score of at least 0.8 were fed into MatDefine (Genomatix software package) to create  
171 a highly conserved matrix for each transcription factor binding site. The settings were as follows:  
172 tuple size – 8; no. of sequences containing tuple – 60%; matrix similarity score for sequence  
173 inclusion – 0.9. Matrices for GAS, ISRE and NF $\kappa$ B binding sites were then used in pSCAN as  
174 «user supplied matrices» to search for occurrences in genes two-fold upregulated in the  
175 atherosclerotic plaque datasets.

176 **ChIP-qPCR**

177 ChIP was performed as previously described [30] with minor modifications. Briefly, VSMCs  
178 were stimulated as depicted in Fig. 1 and next crosslinked with 1% formaldehyde for 10 minutes.  
179 After fixation chromatin was sonicated with a Diagenode Bioraptor to generate 200-1000bp  
180 fragments. Chromatin was immunoprecipitated with pre-immune IgG (Millipore, 12–371B), or

181 with an antibody against STAT1 (Santa Cruz, sc-346) or IRF1 (Santa Cruz, sc-13041X) or NFκB  
182 (Santa Cruz, sc-398442X). Chromatin-antibody complexes were precipitated with agarose beads  
183 according to the EZ ChIP protocol ( Millipore, 17-371 ). After DNA fragments were column  
184 purified DNA concentration was measured with a Qubit fluorometer. Immunoprecipitated DNA  
185 was quantified by qPCR (primers for Cxcl10: 5'-CCTGTAAACCGAGGGCATTG-3', 5'-  
186 CACGCTTTGGAAAGTGAAC-3') and normalized to values obtained after amplification of  
187 unprecipitated (input) DNA.

#### 188 **Western blot analysis.**

189 Total IRF8, STAT1 (Santa Cruz, sc6058, sc346), GAPDH and phosphorylated STAT1 (Cell  
190 Signaling, 5174s, 91711) were determined by western blotting in VSMCs and HMECs. After  
191 treatment cells were homogenized in a Ripa lysis buffer (Sigma) containing phosphatases and  
192 proteases inhibitors (Roche). Protein concentration was determined using a bicinchoninic acid  
193 protein assay kit (Thermo Fisher Scientific). 40μg of protein per lane was loaded and resolved by  
194 SDS-poly-acrylamide gel electrophoresis (PAGE) under reducing conditions. Proteins were  
195 transferred onto PVDV (Millipore) membrane. After incubation with primary and horseradish  
196 peroxidase-conjugated secondary antibodies (Santa Cruz), immunoreactivity was detected by  
197 adding Luminata Forte Western Substrate (EMD Millipore) and measured by INTAS imaging  
198 system (Intas, Goettingen, Germany).

199

#### 200 **Cytokine detection ELISA.**

201 Expression of murine Cxcl10, Ccl5 (Peprotech) as well as Cxcl9 (Sigma) was performed on  
202 medium remained after treatment of VSMCs using sandwich ELISA tests according to the  
203 manufacturer's instructions.

#### 204 **Measurement of nitric oxide (NO)**

205 VSMCs were treated as depicted in cell experiment section. After treatment medium was  
206 refreshed and cells were cultivated for further 24h. Subsequently medium was collected and  
207 100ul was used to measure amount of NO by Griess diazotization reaction [31]. Medium was  
208 incubated with freshly prepared solution containing 1% sulfanilamide 5% HCl, 0.1% aqueous  
209 solution of 2-(1-Naphthylamino)ethylamine dihydrochloride (Sigma). After 10min incubation  
210 OD at 560nm was measured and compared to the standard curve.

211

#### 212 **Migration assay**

213 Migration assay was performed according to Guo et al [32]. Briefly,  $10^6$  of isolated red blood  
214 cells depleted splenocytes isolated from WT mice, were loaded into the upper chamber of  
215 Transwell 24-well plates (Corning, 3421). The bottom chamber was filled with 600ul of the  
216 medium collected after treatment of VSMCs with LPS, IFN $\gamma$  or IFN $\gamma$  and LPS. After incubation  
217 for 3h at 37°C, migrated cells were stained with CD45FITC and CD3APC antibody (Miltenyi  
218 Biotec 130091609, 130092977) and analyzed by flow cytometer (Miltenyi Biotec).

219

#### 220 **Ex vivo contractility studies**

221 Isolated aortas were cleaned from perivascular fat, cut into 2mm long rings (for myograph) and  
222 placed in a DMEM medium containing 2% FBS (Sigma). Next, aortas were treated with 10ng/ml  
223 of IFN $\gamma$  and/or 1 $\mu$ g/ml of LPS. Vascular contractility studies were performed according to the  
224 technique described by Mulvany et al. [33]. After treatment, 2mm long rings were mounted in a  
225 4-channel myograph (620M, Danish Myo Technology, Aarhus, Denmark) in the organ chamber  
226 filled with physiological saline solution (PSS) containing 118.99mM NaCl, 4.69mM KCl,  
227 1.17mM MgSO $_4$ \*7H $_2$ O, 1.18mM KH $_2$ PO $_4$ , 2.5mM CaCl $_2$ \*2H $_2$ O, 25mM NaHCO $_3$ , 0.03mM  
228 EDTA, 5.5mM Glucose. During the experiment PSS buffer was aerated with carbogen (95% O $_2$ +  
229 5%CO $_2$ ). After calibration, vessels were pre-stretched to obtain optimal passive tension. Next,  
230 vascular functions were analyzed. Contractility was evaluated by substitution of PSS buffer for  
231 high potassium physiological saline solution (KPSS; 74.7mM NaCl, 60mM KCl, 1.17mM  
232 MgSO $_4$ \*7H $_2$ O, 1.18mM KH $_2$ PO $_4$ , 1.6mM CaCl $_2$ , 14.9mM NaHCO $_3$ , 0.026mM EDTA, 5.5mM  
233 Glucose). For testing viability, vessels were subjected to noradrenaline-induced constriction  
234 followed by acetylcholine dilation (Sigma). After washing out with PSS buffer and resting for 15  
235 minutes, noradrenaline dose-response curves was performed. Noradrenaline was used in stepwise  
236 increased, cumulative concentration ranging from  $10^{-11}$  to  $10^{-6}$  mol/L. To study vasodilatation,  
237 sodium nitroprusside (Sigma) was used in concentrations from  $10^{-10}$  to  $10^{-5}$  mol/L

#### 238 **Histology and immunohistochemistry**

239 Histological analyses and immunohistochemistry were performed on representative sections (2-3  
240  $\mu$ m) of formalin fixed in paraffin embedded tissue samples from six human carotid

241 atherosclerotic lesions and four healthy controls. The human tissue samples used in our study  
 242 were procured from Biobank of Department of Vascular and Endovascular Surgery (Klinikum  
 243 rechts der Isar der Technischen Universitaet Muenchen). Collecting of specimens for the  
 244 mentioned Biobank was approved by the local ethics committee (Ethikkommission der Fakultaet  
 245 fuer Medizin der Technischen Universitaet Muenchen) and written informed consent was given  
 246 by all patients. Haemalaun-Eosin (HE) and Elastica-van-Gieson (EvG) staining were performed  
 247 in order to assess sample morphology. For characterisation of the cells within atherosclerotic  
 248 plaques, specimens were treated with antibodies against vascular smooth muscle cells (smooth  
 249 muscle myosin heavy chain 1 and 2 (SM-M10), rabbit polyclonal, dilution 1:4.000 (Abcam ,  
 250 ab81031) and endothelial cells (anti-CD31, mouse monoclonal, clone JC70A, dilution 1:100;  
 251 Dako).

252 For the detection of specific cytokines, CXCL9 (MIG) and CXCL10 (IP10), as well as the  
 253 phosphorylated transcription factor STAT1, following primary antibodies were used: rabbit  
 254 polyclonal anti-MIG (Abcam, ab9720; dilution 1:500), rabbit polyclonal anti-IP10 (Abcam,  
 255 ab47045; dilution 1:200), and rabbit monoclonal phospho-Stat1 (Cell Signaling, 91711; dilution  
 256 1:400). All antibodies were first optimised on tonsil using different dilutions, staining conditions  
 257 and with or without blocking. Optimal results were achieved by blocking anti-MIG and anti-  
 258 phospho-Stat1 with goat serum, anti-IP10 without the blocking procedure.

259 Following incubation with primary antibody visualisation was performed by peroxidase/DAB  
 260 ChemMate Detection Kit according to the manufacturer's instruction (biotinylated goat anti-  
 261 mouse/anti-rabbit secondary Ab; Dako).

## 262 **Statistical Analysis**

263 Data are presented as mean  $\pm$  SEM. For comparisons between more than two groups one-way  
 264 ANOVA with Tukey post-hoc test was used. In all other experiments comparing two groups,  
 265 Student's t-test was used. A probability value  $<0.05$  was considered statistically significant  
 266 (GraphPad Prism ® 5.0). In contractility studies, two-way ANOVA test with Bonferroni post hoc  
 267 test was used.

268

269



## 270 Results

### 271 IFN $\gamma$ and LPS synergistically induce CXCL10 expression in VSMCs, depending on STAT1

272 Recently, we showed that in ECs cross-talk between IFN $\gamma$  and TLR4 resulted in augmented  
273 STAT1 phosphorylation and increased expression of the chemokine CXCL10 [17]. To study if a  
274 similar mechanism affected the expression of Cxcl10 in VSMCs, these cells were isolated from  
275 WT and STAT1<sup>-/-</sup> mice and treated as depicted in Fig. 1. In WT-VSMCs, treatment with IFN $\gamma$  or  
276 LPS alone induced expression of Cxcl10 at the mRNA (Fig. 1A) as well as at the protein level  
277 (Fig. 1B). Furthermore, pre-treatment with IFN $\gamma$  for 4h followed by LPS for another 4h led to  
278 synergistic amplification of Cxcl10 expression compared with both factors alone (Fig 1A and  
279 1B). In contrast, this IFN $\gamma$  and LPS-induced synergistic amplification in Cxcl10 gene expression  
280 was dramatically abrogated in STAT1<sup>-/-</sup>-VSMCs (Fig. 1A), which coincided with Cxcl10 protein  
281 levels in the medium (Fig. 1B) and correlated with a predominant STAT1-dependent mechanism.

### 282 Transcriptional responses in IFN $\gamma$ and LPS treated VSMCs predict dependence on STAT1, 283 NF $\kappa$ B and IRF

284 Next, we compared genome-wide transcriptional responses of WT-VSMCs to LPS (4h) or IFN $\gamma$   
285 (8h) alone, or after combined treatment (IFN $\gamma$  8h, LPS 4h). IFN $\gamma$  changed the expression of 297  
286 and LPS of 553 genes under these conditions (Fig. 2A). The interactions between IFN $\gamma$  and LPS  
287 (Fig. 2A) increased the number of up-regulated genes to 990. While 128 of the IFN $\gamma$ -regulated  
288 genes were modulated by LPS (Fig. 2A), 118 were also commonly regulated by IFN $\gamma$  + LPS.  
289 Likewise, we compared transcriptional responses of STAT1<sup>-/-</sup>-VSMCs to LPS or IFN $\gamma$  alone, or  
290 after combined treatment. Only 16 genes were found to be up-regulated by IFN $\gamma$  in STAT1<sup>-/-</sup>-  
291 VSMCs, highlighting the importance of STAT1 in this response pathway. In contrast, LPS  
292 treatment of STAT1<sup>-/-</sup>-VSMCs was similar to WT-VSMCs, with a total of 470 genes being  
293 modulated. However, in general the potency of the response was lower as compared to WT-  
294 VSMCs. Consequently, the additive or synergistic effect of IFN $\gamma$  and LPS as seen in WT-VSMCs,  
295 was no longer present in STAT1<sup>-/-</sup>-VSMCs. Only 493 genes were upregulated by IFN $\gamma$  + LPS, of  
296 which 323 were in common with LPS alone. The complete list of up and down-regulated genes  
297 in response to IFN $\gamma$  or LPS alone, or after combined treatment is shown in Suppl. Table I, II and  
298 III, respectively.

299 Subsequently, we aimed at identifying the genes, that similar to Cxcl10 were synergistically  
 300 affected by the interactions between IFN $\gamma$  and LPS, and their dependency on STAT1. Table I  
 301 shows the top 30 synergistically amplified genes of which the expression was at least 2-fold  
 302 higher upon stimulation with IFN $\gamma$  + LPS as compared to the sum of the treatments with both  
 303 factors alone. For example, expression of Cxcl9 was >15-fold higher after combined treatment  
 304 [2643.5-fold increased by IFN $\gamma$  + LPS, divided by 171 times as the sum of IFN $\gamma$  (150.73) and  
 305 LPS (20.25) alone] as compared to the single treatments.  
 306 Subsequently, by grouping these 30 genes based on their response pattern in WT-VSMCs to IFN $\gamma$   
 307 or LPS (Fig. 2B), we could distinguish five groups of genes (Fig. 2C). The first group contained  
 308 genes with high response to both IFN $\gamma$  and LPS that were highly amplified after combined  
 309 treatment (Cluster A in Table I and Fig. 2C). These include Cxcl9, Cxcl10, Rsad2 and Gbp5. The  
 310 expression of a second group of genes, including Batf2, Ubd, Cd74, Fam26f and Serpina3g,  
 311 showed high response to IFN $\gamma$ , mild response to LPS, and high amplification after combined  
 312 treatment (Cluster B in Table I and Fig. 2C). In contrast, a third group of genes showed a mild or  
 313 no response to IFN $\gamma$ , high response to LPS, and again high amplification after combined  
 314 treatment (Cluster C in Table I and Fig. 2C). This group was exemplified by Ccl5, Tnfaip2,  
 315 Cd40, LincR and Nos2 (iNOS). The fourth group of genes consisted of Ccr12, Mx1, Has1, Oasl1,  
 316 MCP-2, Atf3, Ifi205, Upp1 and Tnfrsf11a and displayed mild or no response to IFN $\gamma$ , mild  
 317 response to LPS, and mild amplification after combined treatment (Cluster D in Table I and Fig.  
 318 2C). Finally, we could also identify genes which showed minor or no response to IFN $\gamma$  and LPS  
 319 alone, but were highly amplified in expression after combined treatment [e.g., Irf8, MCP-5,  
 320 Sectm1a, Gja4, Egr2, Itpk1 and Etsrp71] (Cluster E in Table I and Fig. 2C).  
 321 In general, the absence of STAT1 severely abrogated the IFN $\gamma$ -induced expression of all of these  
 322 30 genes. On the other hand, the LPS response of 50% of genes listed in Table I, was decreased  
 323 in the absence of STAT1. Consequently, the synergistic effect of IFN $\gamma$  and LPS as seen in WT-  
 324 VSMCs, was no longer present in STAT1<sup>-/-</sup>-VSMCs. As the only exception, the IFN $\gamma$ -induced  
 325 expression of MCP-2 appeared STAT1-independent, with a similar fold induction in WT and  
 326 STAT1<sup>-/-</sup> VSMCs (Table I, 6.12 vs. 5.12). In contrast, absence of STAT1 increased its response  
 327 to LPS (Table I, 3.75 vs. 44.53).

328 Successive promoter analysis of the genes listed in Table I, predicted the presence of STAT-  
 329 NFκB and IRF-NFκB modules or combinations of separate ISRE, STAT or NFκB binding sites,  
 330 strongly implicating the cooperative involvement of NFκB, STAT1 and/or IRFs in the  
 331 transcriptional regulation of all of these genes in response to IFNγ and LPS.

332

333 **Transcriptional responses in IFNγ and LPS treated VSMCs and ECs predict a pro-**  
 334 **atherogenic phenotype**

335

336 Gene ontology (GO) functional analysis of the top 30 genes listed in Table I, revealed significant  
 337 enrichment in biological functions involved in host defense, immune response, inflammatory  
 338 response, cytokine response, response to stress and to wound healing (Table II). All these  
 339 categories generally recognize a similar group of genes, including the chemokines Cxcl9, Ccl12,  
 340 Ccl8, Ccl5, Cxcl10 and Ccr12, adhesion molecules (Cd40, Cd74), and the antiviral and  
 341 antibacterial response genes Irf8, Rsad2, Mx1, Oasl1, Gbp5, Nos2, Batf2 and Tnfrsf11a.  
 342 Together, these genes reflect an enhanced pro-inflammatory and pro-atherogenic profile that is  
 343 mediated by interactions between IFNγ and LPS in VSMCs and strongly depends on STAT1.

344 The expression of the chemokines Ccl5, Cxcl9, Ccl12 and chemokine receptor Ccr12 (not shown)  
 345 was additionally examined by qPCR and ELISA (Fig. 3), and confirmed the microarray data. In  
 346 agreement with Table I, the response of this selected group of genes was severely abolished in  
 347 STAT1<sup>-/-</sup>-VSMCs, confirming the importance of STAT1 in the signal integration between IFNγ  
 348 and LPS. Because we were not able to isolate a homogeneous population of mouse aortic  
 349 endothelial cells (data not shown), we instead used the human microvascular endothelial cell-  
 350 line, HMEC [20]. Pre-treatment of HMECs with IFNγ for 4h followed by LPS for another 4h  
 351 resulted in a similar amplification pattern of Ccl5, Cxcl9 and Cxcl10 (Fig. 3C) as in WT-VSMCs,  
 352 providing evidence for a universal STAT1-dependent mechanism in vascular cells triggered by  
 353 IFNγ and LPS. Similarly, we were able to observe a synergistic amplification after IFNγ and  
 354 LPS treatment of Cxcl9 and Cxcl10 in ex vivo treated aortic rings of WT animals as compared to  
 355 IFNγ or LPS alone (Fig 3D)

356 Finally, Chromatin-immunoprecipitation (ChIP)-qPCR of untreated WT-VSMCs or treated with  
 357 IFNγ, LPS or IFNγ + LPS and using antibodies against STAT1 NFκB, IRF1 or IgG, clearly

showed enhanced binding of these different transcription factors to the ISRE and NFκB binding elements of the Cxcl10 gene, as compared to IgG controls (Fig. 3E). In a representative experiment, STAT1 binding to the ISRE increased after IFNγ as well as LPS treatment, but not after IFNγ + LPS stimulation. IRF1 binding was enriched upon treatment with IFNγ alone and after subsequent stimulation with LPS. LPS alone, on the other hand did not affect IRF1 binding. Finally, NFκB binding dramatically increased when cells were first treated with IFNγ and then by LPS, but not in the presence of IFNγ or LPS alone (Fig. 3E). This confirms the cooperative involvement of STAT1, NFκB and IRF1 in the transcriptional regulation of Cxcl10 in response to IFNγ and LPS as predicted in Table I.

#### **IRF8 mediates IFNγ and LPS induced Ccl5 expression in vascular cells.**

Notably, the transcription factor IRF8, which was thought to be restricted to lymphoid-cell lineages such as B-, T-, dendritic cells and macrophages, was identified among the amplified genes. Indeed, gene (Fig. 4A left panel) and protein expression (Fig. 4B left panel) of IRF8 in WT and STAT1<sup>-/-</sup> VSMCs in response to IFNγ, LPS or IFNγ + LPS, confirmed the microarray data. Interestingly, pre-treatment of ECs with IFNγ for 4h followed by LPS for another 4h resulted in a similar amplification pattern of IRF8 RNA (Fig. 4A right panel) and protein expression (Fig. 4B right panel) as in WT-VSMCs. These results provide evidence for STAT1-dependent expression of IRF8 in VSMCs and ECs upon treatment with IFNγ, and confirm amplification of IRF8 upon stimulation with IFNγ and LPS in vascular cells.

Next, the IRF8 dependent regulation of Ccl5 (a known IRF8 target in immune cells [34]) was examined. The amplified expression of Ccl5 RNA (Fig. 4C, left panel) and protein (Fig. 4C, right panel) in response to IFNγ and LPS, as seen in WT VSMCs, was highly attenuated in IRF8<sup>-/-</sup> and STAT1<sup>-/-</sup>-VSMC. In contrast, the expression of Cxcl10 and Cxcl9 in response to IFNγ and LPS in WT VSMCs was similar to that in IRF8<sup>-/-</sup>-VSMCs (Fig. 4D).

#### **Signal integration between IFNγ and LPS in VSMCs leads to increased migration of T-lymphocytes**

Since many of the chemokines characterized above are involved in chemotaxis of T-lymphocytes [35], we examined the effect of IFNγ and LPS cross-talk on T-cell migration towards conditioned medium from treated VSMCs. Amplification of chemokines in WT-VSMCs upon stimulation with both stimuli indeed led to an increased migration of CD3<sup>+</sup>/CD45<sup>+</sup> splenocytes

(Fig. 4E). Migration of CD3<sup>+</sup>/CD45<sup>+</sup> cells towards medium of WT-VSMCs treated with both IFN $\gamma$  and LPS was significantly higher (234%) as compared to both factors alone (125% and 175%, respectively). As expected, the chemotactic response of splenocytes towards the conditioned medium obtained after treatment of STAT1<sup>-/-</sup>-VSMCs was highly attenuated (Fig. 4E).

#### **Signal integration between IFN $\gamma$ and LPS in aortic rings leads to abolished response to norepinephrine and sodium nitroprusside.**

Among the genes that were highly amplified upon treatment with IFN $\gamma$  and LPS was inducible nitric oxide synthase (iNOS, Nos2). Indeed, treatment of WT-VSMCs but not STAT1<sup>-/-</sup> with IFN $\gamma$  and LPS caused amplified expression of Nos2 as compared to stimulation with both factors alone (Fig. 5A). The RNA levels reflected nitrite accumulation in the medium (Fig. 5B). Since dysregulation of Nos2 expression and its activity affects vessel function, we evaluated the physiological ramifications of these experimental conditions using a wire myograph/organ chamber setting. Stimulation of the aortic rings isolated from WT animals with IFN $\gamma$  and LPS resulted in drastic impairment of contractility after subjection to norepinephrine treatment (Fig. 5C, left panel). WT vessels treated with both IFN $\gamma$  and LPS manifested also high loss of the sensitivity to sodium nitroprusside (Fig. 5D, left panel). In contrast to WT, aortic rings from STAT1-deficient mice did not reveal ameliorated response to noradrenaline and sodium nitroprusside as compared to LPS stimulated vessel (Fig. 5C, Fig 5D, right panel).

#### **STAT1 activation and CXCL9 and CXCL10 expression in ECs and VSMCs from human carotid atherosclerotic plaques**

We performed immunohistochemistry staining for phosphorylated STAT1, CXCL9 and CXCL10 in human advanced atherosclerotic plaques of carotid arteries in comparison to healthy vessels. As can clearly be observed in Figure 6A, VSMCs in the lesion highly expressed phosphorylated STAT1 and both chemokines CXCL9 and CXCL10. In contrast, healthy vessels were negative for all three markers (Figure 6A). Moreover, ECs covering the plaque likewise showed predominant staining for phosphorylated STAT1 and CXCL9, and to a lesser extent for CXCL10 (Figure 6B). Again, healthy endothelium was negative. Staining for IRF8 was more difficult to interpret, but seemed present at low levels in SMCs (not shown).

420 **STAT1-dependent pro-atherogenic target gene expression in human atherosclerotic**  
421 **plaques**

422 To obtain potential evidence for STAT1-mediated target gene expression in the human  
423 atherosclerotic plaque, we performed different types of experiments. First, we analyzed two  
424 independent microarray datasets obtained from human coronary plaques and human carotid  
425 plaques. These datasets are available in GEO NCBI database (acc. no. GSE40231 and  
426 GSE21545, respectively) [26,27]. In coronary and carotid plaques respectively we identified  
427 1146 and 949 genes upregulated at least 1.5 times as compared to the healthy arterial tissue  
428 (Figure 7A). 201 of those genes are commonly expressed between the different plaque tissues,  
429 highly implying that there are common features between coronary and carotid plaques (Sikorski  
430 et al. [36]). Detailed promoter analysis of the differentially expressed genes in carotid and  
431 coronary plaques identified 128 (Figure 7B) and 362 (Figure 7C) genes, respectively containing  
432 GAS, ISRE or NFκB sites, either alone or in different combinations. This strongly suggests also  
433 the cooperative involvement of NFκB, STAT1 and/or IRF in the transcriptional regulation of  
434 genes in the plaque tissue.

435 Next, we compared the 30 IFNγ and LPS amplified STAT1-target genes listed in Table I to the  
436 genes upregulated in carotid and coronary plaques. Indeed, 12 out of the 30 genes were  
437 expressed in carotid plaques and 6 out of 30 in coronary plaques, including CXCL9, CXCL10,  
438 CCL5, CCL8, CRCL2, Cd74, GBP5, UBD, SECTM1, IFI16 (homologue Ifi-205), UPP1,  
439 FAM26F and the transcription factor IRF8 as the above identified STAT1 targets (Fig. 7A).

440 Together, this points to a pro-atherogenic role of STAT1 in vascular cells of atherosclerotic  
441 plaques, and suggests the potential of a selection of STAT1-target genes as biomarkers to  
442 monitor plaque phenotype in human atherosclerosis.

443

444

445

446

## 447 Discussion

448 The involvement of STAT1 in experimental atherosclerosis has recently been appreciated,  
449 especially in immune cells. It is additionally accepted that in immune cells STAT1 is a unique  
450 point of convergence for the antimicrobial and inflammatory synergism between IFN $\gamma$  and  
451 TLRs. Recently, we showed that also in ECs cross-talk between IFN $\gamma$  and TLR4 resulted in  
452 augmented STAT1 phosphorylation and increased expression of the chemokine CXCL10 [17].  
453 Here, a similar STAT1-dependent mechanism for CXCL10 expression in response to IFN $\gamma$  and  
454 LPS was observed in VSMCs (Fig. 1). To date, no information is available on the genome-wide  
455 induced changes modulated by IFN $\gamma$  and TLR4 in ECs and VSMCs and how this affects vascular  
456 function. Therefore, we decided to further characterize the role of STAT1 in the transcriptional  
457 response pathways involved in the interaction between IFN $\gamma$  and TLR4 signaling in VSMCs.  
458 Thus, we identified a specific set of STAT1-dependent genes that were synergistically affected  
459 by IFN $\gamma$  and LPS in VSMCs in vitro. These included the chemokines Cxcl9, Ccl12, Ccl8, Ccl5,  
460 Cxcl10 and Ccr12, adhesion molecules (Cd40, Cd74), and the antiviral and antibacterial response  
461 genes Irf8, Rsad2, Mx1, Oasl, Gbp5, Nos2, Batf2 and Tnfrsf11a. Based on their response pattern  
462 to IFN $\gamma$ , LPS and IFN $\gamma$  + LPS in WT-VSMCs (Fig. 2B), we could distinguish five clusters of  
463 genes (Fig. 2C). In general, the absence of STAT1 severely abrogated the IFN $\gamma$ -induced  
464 expression of all of these genes. Moreover, the LPS response of 50% of genes listed in Table I  
465 was decreased in the absence of STAT1. Consequently, the synergistic effect of IFN $\gamma$  and LPS,  
466 as seen in WT-VSMCs, could no longer be detected in STAT1<sup>-/-</sup>-VSMCs. This strongly suggests  
467 the involvement of STAT1 in the signal integration between JAK/STAT and TLR4 pathways.  
468 The IFN $\gamma$ -induced expression of Ccl8 appeared STAT1-independent, with a similar fold  
469 induction in WT and STAT1<sup>-/-</sup> VSMCs. In contrast, absence of STAT1 increased its response to  
470 LPS. As Ccl8 is a known STAT3 target gene [37], it is possible that it's IFN $\gamma$  and LPS  
471 inducibility in WT and STAT1<sup>-/-</sup> VSMCs, is regulated by this transcription factor. The increased  
472 LPS-mediated Ccl8 expression in STAT1<sup>-/-</sup> VSMCs as compared to WT cells, could potentially  
473 be explained by the absence of a STAT1-dependent inhibitory mechanism of STAT3 activity  
474 mediated by the STAT1-target gene SOCS1.

475 The expression of a selection of these genes, including Ccl5, Cxcl9, Nos2, Irf8 and Ccl12, Ccr12  
476 (not shown) was additionally determined at the RNA and protein level, and confirmed the  
477 microarray data. A similar expression pattern of some of these genes could also be identified in

478 ECs and aortic ring segments, providing evidence for a universal STAT1-dependent mechanism  
479 in vascular cells triggered by IFN $\gamma$  and LPS.

480

481 Integration of IFN $\gamma$  and TLR signaling pathways occurs, for instance, through synergy between  
482 TLR- and IFN $\gamma$ -induced transcription factors. Promoter analysis of the genes listed in Table I  
483 indeed predicted the presence of STAT-NF $\kappa$ B and IRF-NF $\kappa$ B modules or combinations of  
484 separate ISRE, STAT or NF $\kappa$ B binding sites in their promoters. Indeed, ChIP-qPCR confirmed  
485 binding of STAT1, NF $\kappa$ B and IRF1 to the Cxcl10 gene, in an IFN $\gamma$  and LPS-dependent manner  
486 (Figure 3E). This strongly suggested that cooperation between NF $\kappa$ B, STAT1 and/or IRFs is  
487 involved in the transcriptional regulation of all of these genes in response to IFN $\gamma$  and LPS.  
488 According to previous studies, transcription of genes that contain STAT1- and NF $\kappa$ B-binding  
489 sites in their promoter regions are often cooperatively regulated by extracellular stimuli that  
490 induce STAT1 and NF $\kappa$ B, such as IFN $\gamma$  and TNF $\alpha$ , IL-1 $\beta$  or LPS [38,39,40,41,42,43]. Likewise,  
491 genes with both an ISRE element and NF $\kappa$ B-binding site are subjected to a similar mechanism of  
492 signal integration [44,45]. In general it is believed that in immune cells, multiple inflammatory  
493 stimuli culminate in gene expression that requires cooperation between NF $\kappa$ B and STAT1 or  
494 NF $\kappa$ B and IRF1 [7]. They ultimately promote type I immune actions, which are associated with  
495 host-defense mechanisms against viral and bacterial infections and excessive immune responses  
496 [46]. Our data provides strong evidence that a similar mechanism of signal integration exists in  
497 vascular cells. The difference in expression pattern of these 30 genes did not correlate with the  
498 presence of a specific binding site or combination of binding sites. This implies that the affinity  
499 of the different transcription factors is most likely determining the transcriptional response of a  
500 particular gene.

501 Among the amplified genes we also identified the transcription factor IRF8, which expression is  
502 thought to be restricted to lymphoid-cell lineages such as B-, T- and macrophages. Thus, IRF8  
503 may in part account for “immune cell-specific” STAT1-dependent functions of IFN $\gamma$ . IRF8 is  
504 also directly connected to TLR action, regulating the production of type I IFNs and other  
505 inflammatory mediators. For example, co-administration of IFN $\gamma$  and LPS to macrophages  
506 caused super-induction of IRF8 and IRF8 target genes[47]. As a consequence, synergistic  
507 induction of the pro-inflammatory genes IL1, IL6, IL12 and TNF $\alpha$  was observed in an IRF8  
508 dependent manner. In addition to its known immune cell functions, our results now uncover a



509 novel “inflammation-dependent” role of IRF8 in cells from the vasculature. In ECs as well as  
 510 VSMCs, combined treatment of IFN $\gamma$  and LPS resulted in a synergistic increase in IRF8 gene  
 511 and protein expression as compared to both factors alone (Figure 4A and B). The presence of a  
 512 potential STAT1-NF $\kappa$ B module in the IRF8 promoter (Table I) highly suggests that the  
 513 cooperation of these two transcription factors is at the basis of its synergistic expression.  
 514 Consequently, this revealed the possible existence of IRF8-dependent cross-talk between IFN $\gamma$   
 515 and LPS in vascular cells. Indeed, we subsequently identified Ccl5 (but not Cxcl9 and Cxcl10) as  
 516 a novel IRF8 target in VSMCs and ECs (Fig. 4C and D). The transcriptional regulation of the  
 517 Ccl5 gene in macrophages in response to IFN $\gamma$  and LPS, was recently shown to involve IRF8 in  
 518 combination with IRF1 and NF $\kappa$ B[34]. Therefore, the IRF8-dependent expression of Ccl5 in  
 519 IFN $\gamma$ -primed vascular cells that are subsequently stimulated by LPS is highly likely to comprise  
 520 a similar mechanism. These results correlates with the predicted presence of an IRF-NF $\kappa$ B  
 521 module in the Ccl5 promoter (Table I). On the other hand, the same promoter contains also a  
 522 potential STAT1-NF $\kappa$ B module (Table I) which suggests the additional involvement of STAT1  
 523 as well.  
 524 Together, our detailed investigation of STAT1-dependent transcriptional synergism between  
 525 IFN $\gamma$  and LPS in cells from the vasculature predicts the existence of different regulatory  
 526 mechanisms. It particularly involves cooperation between STAT1, IRF1, IRF8 and NF $\kappa$ B, with  
 527 the novel role of IRF8 providing an additional layer to the overall complexity.  
 528  
 529 Functional analysis of the STAT1-dependent genes that were synergistically affected by  
 530 interactions between IFN $\gamma$  and LPS in VSMCs (Table I), revealed significant enrichment in  
 531 biological functions connected to host defense, immune response, inflammatory response,  
 532 cytokine response, response to stress and to wound healing (Table II). All these categories  
 533 generally represent a similar group of genes, which together reflect an enhanced pro-  
 534 inflammatory and pro-atherogenic profile.  
 535 The fact that synergistic interactions between IFN $\gamma$  and LPS in VSMCs resulted in increased  
 536 expression of multiple chemokines, prompted us to investigate T-cell migration. Indeed, a  
 537 significant increase in migration of CD3<sup>+</sup>/CD45<sup>+</sup> splenocytes could be detected towards  
 538 conditioned medium from IFN $\gamma$  and LPS treated WT-VSMCs as compared to that from cells  
 539 treated with single factors. Importantly, splenocytes migration occurred in a STAT1-dependent

540 manner, which correlated with decreased chemokine expression in STAT1<sup>-/-</sup>-VSMCs under these  
 541 conditions (Fig. 4E). Interestingly a subset of these chemokines, including CXCL9, CXCL10,  
 542 CCL5, CCL8 and CCRL2, has been reported to be increased in cells from the vasculature, which  
 543 is in agreement with our results. Moreover, evidence exists that chemokines cooperate in  
 544 leukocyte recruitment to the injured artery during vascular remodeling [35,48,49] and as such are  
 545 involved in the pathogenesis of atherosclerosis.  
 546 To further elucidate the functional role of a cross-talk in the vessel, we performed contractility  
 547 studies. The signal integration between IFN $\gamma$  and LPS in aortic ring segments resulted in  
 548 impaired aortic contractility (Fig. 5) and coincided with a dramatic increase in expression of  
 549 Nos2. Nos2 participates in vascular dysfunction and is associated with progression of  
 550 atherosclerosis [50,51].  
 551 More important, we were able to detect phosphorylated STAT1 in VSMCs and ECs of human  
 552 atherosclerotic plaques (Fig. 6), which correlated with elevated expression of the chemokines  
 553 CXCL9 and CXCL10. Recently, Agrawal et al. [10] showed that STAT1 deficiency reduced foam  
 554 cell formation in an intraperitoneal inflammation model and reduced atherosclerosis in an  
 555 atherosclerosis-susceptible bone marrow transplantation mouse model. In combination with our  
 556 results, this highlights the pro-atherogenic role of STAT1 in cells from the vasculature in human  
 557 vascular disease.  
 558  
 559 Using data mining of human plaque transcriptomes, we were able to show that expression of a  
 560 selection of the above identified STAT1-dependent pro-atherogenic genes was significantly  
 561 increased in human plaques from carotid and coronary arteries (Fig. 7A). Of these, CXCL9,  
 562 CXCL10, CCL5, CCL8, CCRL2, Cd74 and IRF8 have previously been implicated in  
 563 atherosclerosis [52,53]. This is not the case for GBP5, Ubd, SectM1, Ifi16, Upp1 and Fam26F,  
 564 and could therefore represent potential novel biomarkers of atherosclerosis. Moreover, CCL5  
 565 expression was higher in carotid (23.3 fold increase) as compared to coronary (2.9 fold increase)  
 566 plaques, which correlated with IRF8 levels (8.8 fold increase in carotid vs. 1.4 fold increase in  
 567 coronary). Detailed promoter analysis of differentially expressed genes in coronary and carotid  
 568 plaques predicted cooperative involvement of NF $\kappa$ B, STAT1 and/or IRF1 in regulation of their  
 569 expression. This could point to the role of IFN $\gamma$  and TLR4 activation in human atherosclerosis,  
 570 which is in agreement with previous studies [6,54,55]. However, we cannot rule out the

571 contribution of other pro-inflammatory stimuli in the regulation of these genes. Nevertheless, our  
572 data strongly suggest involvement of both STAT1 and IRF8 in the regulation of gene expression  
573 in different cell types present in human atherosclerotic plaques.

574

575 In summary, our findings provide additional evidence to suggest that in ECs and VSMCs  
576 STAT1, in cooperation with IRF1, IRF8 and NFκB, coordinates a platform for cross-talk  
577 between IFNγ and TLR4. This results in an increased pro-inflammatory phenotype and leads to  
578 amplified pro-atherogenic responses in the vasculature. As a consequence, in the presence of  
579 IFNγ and LPS (or any other exogenous or endogenous TLR4 ligands), multiple chemokines,  
580 adhesion molecules and antiviral and antibacterial response proteins can be over-produced in  
581 ECs and VSMCs. This may in turn modulate leukocyte attraction, adhesion and VSMC  
582 proliferation and migration, which are important characteristics of vascular dysfunction and early  
583 triggers of atherosclerosis. As such, a predefined STAT1-target gene signature could be  
584 developed as a novel diagnostic tool to monitor and diagnose plaque phenotype in human  
585 atherosclerosis. In addition, STAT1 represents an interesting novel target of therapeutic  
586 intervention that has a crucial role in mediating the interplay between damaged vessels and host  
587 immunity during the process of atherosclerosis.

588

#### 589 **Acknowledgments**

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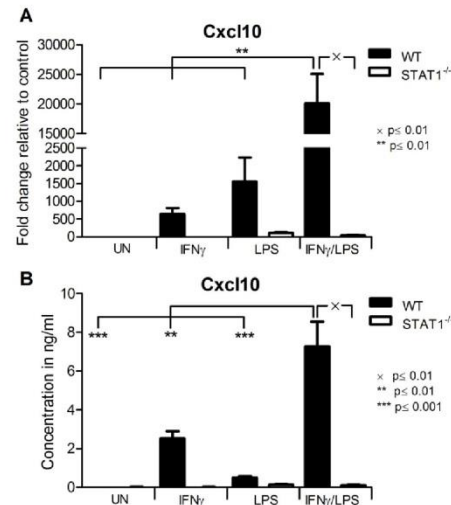
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740 **Figures Legends**



741

742 **Figure 1. CXCL10 amplified by IFN $\gamma$  and LPS in VSMCs is STAT1 dependent.** A, WT and  
 743 STAT1<sup>-/-</sup> VSMCs were treated with 10ng/ml IFN $\gamma$  for 8h or with 1ug/ml of LPS for 4h or with  
 744 IFN $\gamma$  for 4h followed by LPS for additional 4h. RNA was isolated and qRT-PCR for Cxcl10  
 745 using Gapdh as internal control was performed. B, Cells were treated as in A. On the medium  
 746 remained after treatment ELISA for CXCL10 was performed. Data represent means of at least 3  
 747 independent biological experiments  $\pm$ SEM and  $p < 0.05$  was considered as significant. Data were  
 748 tested for significance by one-way ANOVA followed by post-hoc Tukey or unpaired two-tailed  
 749 student T-test when appropriate.

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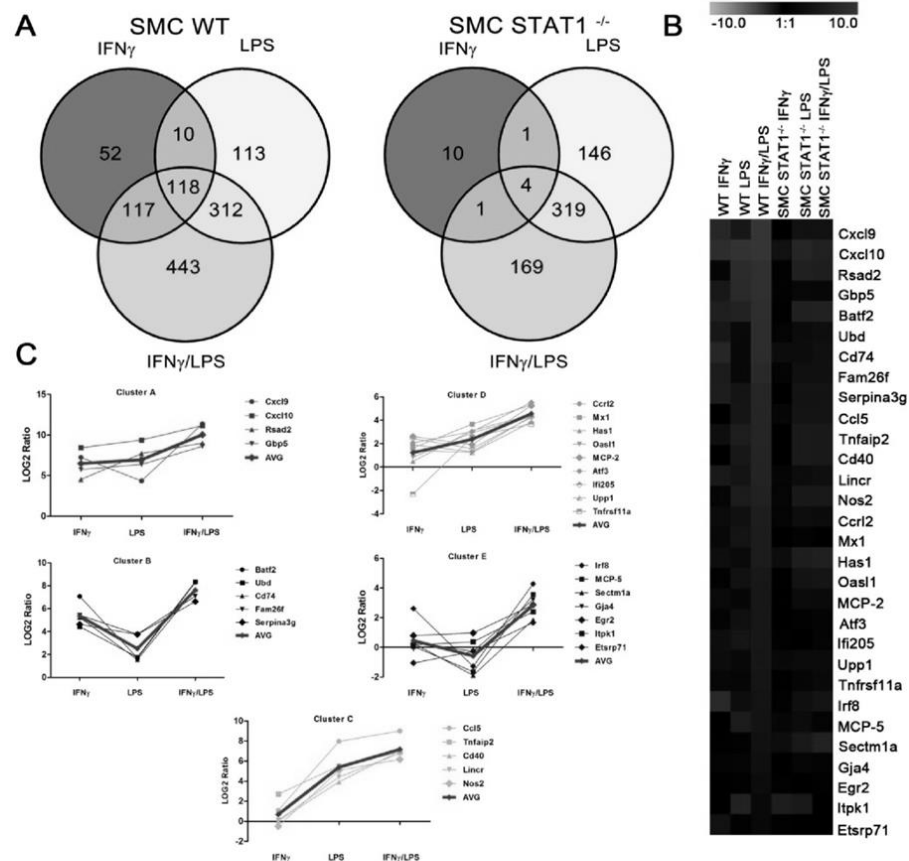
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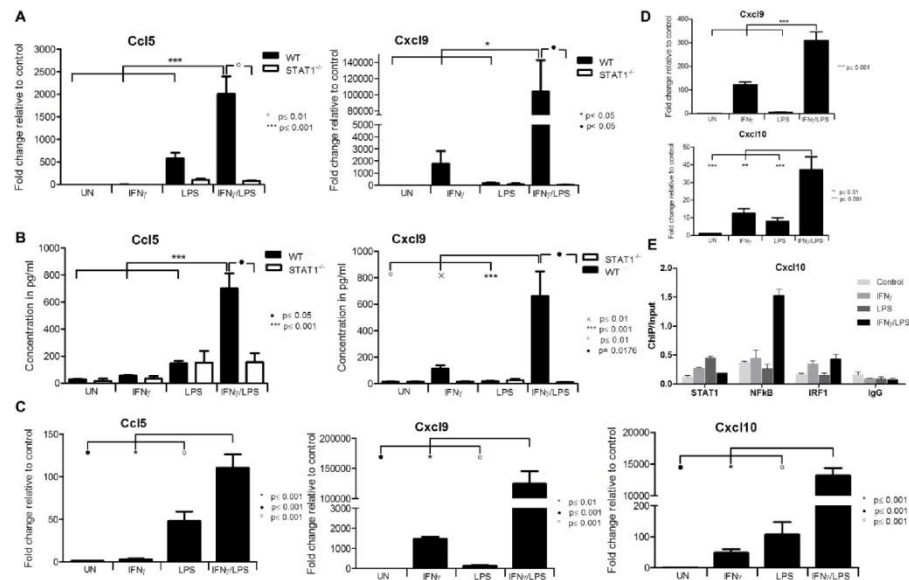
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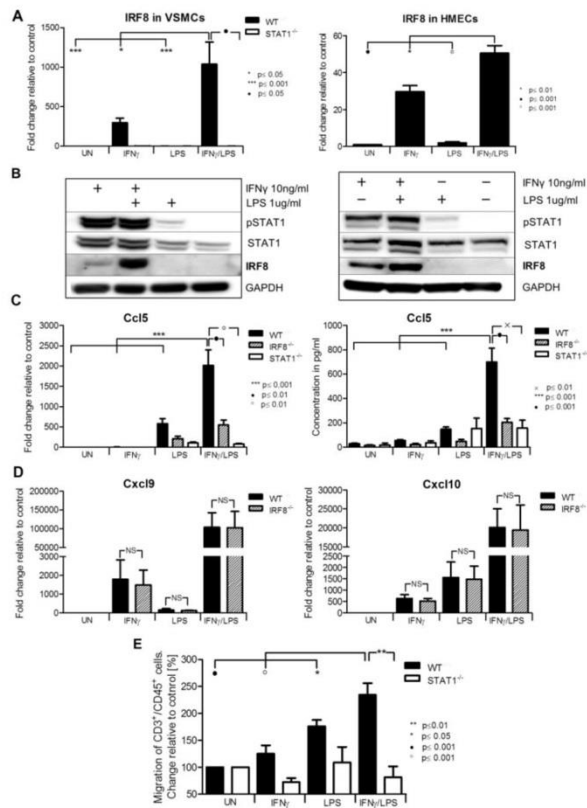


**Figure 2. Identification of genes prone to synergistic amplification upon treatment with IFN $\gamma$  and LPS.** WT and STAT1<sup>-/-</sup> VSMCs were treated as described in Fig 1. On RNA isolated from untreated or IFN $\gamma$ , LPS or IFN $\gamma$  + LPS treated VSMCs genome-wide expression profiling was performed. **A**, Venn diagrams revealing number of differentially expressed genes upon stimulation. **B**, Heat map of the expression of synergistically amplified genes in WT and STAT1<sup>-/-</sup> VSMCs. **C**, Clustering of the synergistically upregulated genes according to their expression profile. AVG, average expression in the group. For details see text.



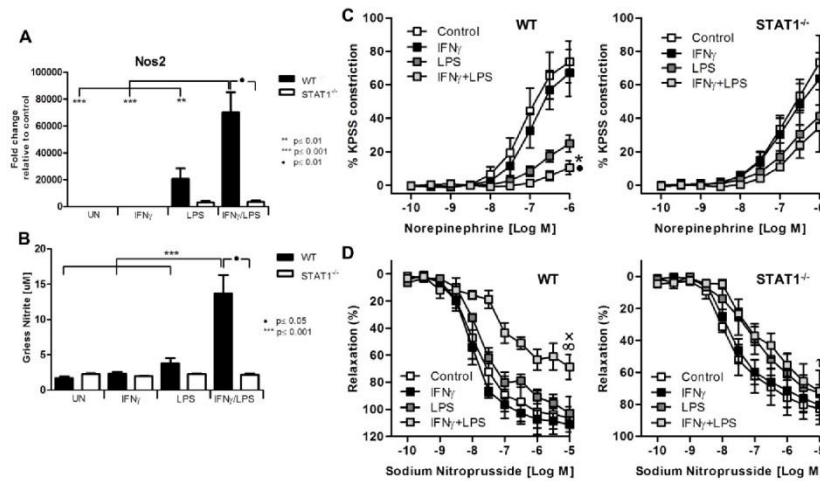


**Figure 3. Effect of STAT1 dependent signal integration on chemokine expression.** WT and STAT1<sup>-/-</sup> VSMCs, HMECs or WT aortic ring segments were treated as described in Fig 1. **A**, RNA from VSMCs was isolated and qRT-PCR for Ccl5, Cxcl9 using Gapdh as internal control was performed. **B**, On the medium remained after treatment of VSMCs ELISA for Ccl5 and Cxcl9 was performed. **C**, Expression of CXCL10, CXCL9 and CCL5 upon stimulation in ECs. **D**, RNA from incubated aortic rings was isolated and qRT-PCR for Cxcl10, Cxcl9 using Gapdh as internal control was performed. Data represent means of at least 3 independent biological experiments  $\pm$ SEM and p<0.05 was considered as significant. Data were tested for significance by one-way ANOVA followed by post-hoc Tukey or unpaired two-tailed student T-test when appropriate. **E**, ChIP-qPCR analysis of the Cxcl10 promoter region containing NFkB and ISRE binding sites show the enrichment with STAT1, NFkB and IRF1 antibodies compared with IgG control in an IFN $\gamma$ , LPS or IFN $\gamma$ +LPS-dependent manner in WT VSMCs. Immunoprecipitated DNA was quantified by qPCR and normalized to values obtained after amplification of unprecipitated (input) DNA. A representative experiment is shown.



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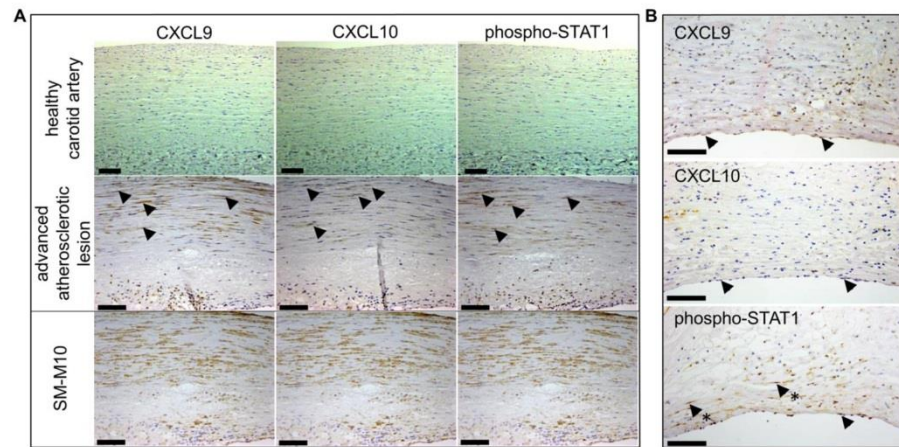
786 **Figure 4. IRF8 mediated cross-talk and functional activity of synergistically amplified**  
 787 **chemokines.** WT, STAT1<sup>-/-</sup> and IRF8<sup>-/-</sup> VSMCs and HMECs were treated as described in Fig 1.  
 788 **A**, RNA was isolated and qRT-PCR for IRF8 using GAPDH as internal control was performed in  
 789 VSMCs (left panel) and ECs (right panel). **B**, Protein extracts were analyzed for IRF8, tyrosine-  
 790 phosphorylated STAT1, total STAT1 and GAPDH. **C**, CCL5 mRNA expression (left panel) and  
 791 protein presence in the medium (right panel) was measured. **D**, Expression profiles of Cxcl9 (left  
 792 panel) and Cxcl10 (right panel) between VSMCs WT, and IRF8<sup>-/-</sup> were compared. **E**, Migration  
 793 assay of CD45<sup>+</sup>/CD3<sup>+</sup> performed on conditioned medium remained after treatment of VSMCs  
 794 WT and STAT1<sup>-/-</sup>. Data represent means of at least 3 independent biological experiments  $\pm$ SEM  
 795 and p<0.05 was considered as significant. Data were tested for significance by one-way ANOVA  
 796 followed by post-hoc Tukey or unpaired two-tailed student T-test when appropriate.



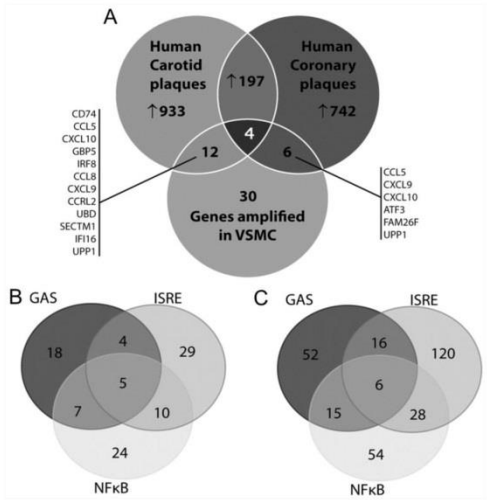
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798 **Figure 5. STAT1-mediated abolished response to norepinephrine and sodium nitroprusside**  
 799 **is associated with disturbed NO production.** A, WT and *STAT1*<sup>-/-</sup> VSMCs were treated as  
 800 described in Fig 1. RNA was isolated and qRT-PCR for *Nos2* using *Gapdh* as internal control  
 801 was performed (upper panel) B, After stimulation as described in Fig. 1, medium was refreshed  
 802 and left for 24h. Next, 100μl of the medium was taken and the product of *Nos2*- nitrite was  
 803 measured. Data represent means of at least 3 independent biological experiments  $\pm$ SEM and  
 804  $p < 0.05$  was considered as significant. Data were tested for significance by one-way ANOVA  
 805 followed by post-hoc Tukey or unpaired two-tailed student T-test when appropriate. C, D  
 806 Isolated aortic rings from WT and *STAT1*<sup>-/-</sup> mice were incubated with 10ng/ml IFN $\gamma$  for 8h or  
 807 with 1μg/ml of LPS for 4h or with IFN $\gamma$  for 4h followed by LPS for additional 4h. Next,  
 808 response to norepinephrine and sodium nitroprusside was tested on the wire myograph. C,  
 809 Response to noradrenaline in WT and *STAT1*-deficient aortic rings presented as a percentage of  
 810 maximal constriction to KPSS. \* $p < 0.001$  vs. WT control; • $p < 0.001$  vs. WT LPS; ○ $p < 0.001$  vs.  
 811 *STAT1*<sup>-/-</sup> control. D, Response to stepwise increased concentration of sodium nitroprusside.  
 812 x $p < 0.05$  vs. WT control; ∞ $p < 0.01$  vs. WT LPS; τ $p < 0.05$  *STAT1*<sup>-/-</sup> control. Aortas isolated from  
 813 3-4 animals per group were taken. Two-way ANOVA test with Bonferroni post hoc test was  
 814 used. Statistical significance for the highest concentration is given.

815



**Figure 6. Expression of pSTAT1, CXCL9, CXCL10 in human atherosclerotic lesions in situ.** Staining of the sections prepared from normal human artery exhibited no presence of pSTAT1, CXCL9, CXCL10 (A, upper panel). In contrast, all three proteins could be detected in SM-M10 positive cells in atherosclerotic plaques (A, middle panel) as well as in the endothelial cells at the lumen side (B). A representative analysis is shown of 6 human carotid atherosclerotic lesions and 4 healthy controls. Arrows represent examples of positive staining. In B arrows with asterisk indicate examples of positively stained VSMCs. Scale bar = 100µm.



**Figure 7. Expression of synergistically amplified genes in atherosclerotic vessels. A,** Venn diagram with analysis of microarray datasets obtained from human coronary plaques and human carotid plaques. **B,** Promoter analysis of the differentially expressed genes in carotid (left panel) and coronary plaques (right panel). For details see text.

**Tables**

**Table I. Genes prone to synergistic amplification upon stimulation with IFN $\gamma$  and/or LPS and their promoter analysis.** The table introduces genes that expression is at least 2-fold higher upon stimulation with IFN $\gamma$ +LPS as compared to the sum of the treatments with both factors alone (see column “Signal integration”). Other numbers represent fold changes compared to control. Cross indicates presence of specific sequence in the promoter regions.

SYMBOL	WT IFN $\gamma$	WT LPS	Signal integration	WT IFN $\gamma$ +LPS	STAT1 <sup>-/-</sup> IFN $\gamma$	STAT1 <sup>-/-</sup> LPS	STAT1 <sup>-/-</sup> IFN $\gamma$ +LPS	STAT_NFkB	IRF_NFkB	ISRE	STAT	NFkB	Cluster
Cxcl9	150.73	20.25	15.46	2643.50	0.93	9.87	9.52	x	-	-	-	-	A
Cxcl10	343.62	665.68	2.25	2273.44	0.47	119.63	66.53	x	x	-	-	-	
Rsad2	22.91	209.45	2.19	509.05	1.53	2.93	2.71	-	-	X	x	x	
Gbp5	53.48	82.66	2.85	388.28	1.28	65.64	72.68	x	-	X	x	x	
Batf2	134.37	3.23	2.29	314.46	5.35	5.10	8.31	x	-	X	x	x	B
Ubd	21.54	3.38	13.11	326.57	0.80	4.71	6.44	x	-	X	-	-	
Cd74	37.66	2.93	5.09	206.50	0.78	8.01	8.24	x	x	-	-	-	
Fam26f	43.02	13.60	2.42	137.16	0.95	13.25	13.07	-	-	X	x	-	
Serpina3g	24.76	13.36	2.59	98.56	2.12	5.12	7.79	x	-	X	-	-	C
Ccl5	2.14	249.59	2.04	512.54	0.83	64.48	52.22	x	x	-	-	-	
Tnfaip2	6.52	45.55	2.27	118.00	1.14	28.83	27.04	x	-	-	-	-	
Cd40	1.03	15.21	7.83	127.14	0.70	14.09	14.14	-	-	X	x	x	
LincR	1.07	21.38	4.84	108.75	1.16	29.33	25.78	-	-	X	x	x	D
Nos2	0.71	33.99	2.08	72.15	0.73	22.52	24.13	x	x	-	-	-	
Ccl2	4.18	8.16	3.72	45.93	0.76	5.87	4.65	x	-	X	-	-	
Mx1	3.10	12.73	2.49	39.41	0.91	1.68	1.18	x	-	X	-	-	
Has1	1.40	8.71	2.16	21.84	1.17	11.64	14.92	-	-	-	x	x	E
Oasl1	1.80	7.61	2.13	20.10	1.03	1.45	1.83	-	-	X	-	x	
MCP-2	6.12	3.75	3.83	37.77	5.21	44.53	58.31	-	-	-	x	-	
Atf3	3.73	2.47	3.27	20.29	1.21	4.34	3.96	-	-	-	x	x	
Ifi205	5.39	2.99	2.28	19.10	0.64	4.24	4.81	-	-	X	x	-	F
Upp1	2.67	2.37	2.87	14.45	1.17	2.25	3.53	x	-	X	-	-	
Tnfrsf11a	0.20	5.87	2.12	12.85	1.46	7.44	12.33	x	-	-	-	-	
Irf8	6.13	0.41	2.98	19.51	1.40	1.03	0.77	x	-	-	-	-	
MCP-5	1.05	0.32	8.65	11.86	0.69	0.59	0.78	-	-	x	x	x	G
Sectm1a	1.21	0.27	2.45	3.62	0.30	0.37	1.16	x	-	x	-	-	
Gja4	1.25	0.83	4.50	9.35	0.55	0.37	0.27	-	-	-	-	x	
Egr2	1.74	1.96	1.98	7.32	0.98	2.31	1.73	x	-	-	-	-	
Itpk1	1.12	1.29	2.19	5.29	0.86	0.97	1.30	-	-	-	x	x	H
Etsrp71	0.48	0.83	2.43	3.18	1.39	0.96	0.96	-	-	-	x	x	

**Table II. Gene ontology classification of synergistically amplified genes**

<b>Term ID</b>	<b>Description</b>	<b>frequency</b>	<b>log<sub>10</sub> p-value</b>	<b>Uniqueness</b>	<b>Dispensability</b>
GO:0051707	response to other organism	0.01	-10.10	0.56	0.00
GO:0009607	response to biotic stimulus	0.01	-9.62	0.66	0.40
GO:0006952	defense response	0.01	-8.91	0.63	0.41
GO:0002376	immune system process	0.01	-7.61	0.97	0.00
GO:0071345	cellular response to cytokine stimulus	0.00	-6.87	0.52	0.32
GO:0006950	response to stress	0.04	-6.59	0.61	0.50
GO:0006955	immune response	0.01	-6.26	0.41	0.39
GO:0006954	inflammatory response	0.00	-5.91	0.68	0.49
GO:0045071	negative regulation of viral genome replication	0.00	-5.04	0.76	0.47
GO:0009611	response to wounding	0.00	-4.93	0.68	0.53